



## **Adaptation of *Campylobacter jejuni* to oxidative stress and poultry meat environment**

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FACULTY OF LIFE SCIENCES  
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# Adaptation of *Campylobacter jejuni* to oxidative stress and poultry meat environment

PhD thesis

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Thesis submitted: September 2011

## **Preface**

This PhD thesis is submitted as one of the requirements for obtaining the PhD degree from the Faculty of Life Sciences at the University of Copenhagen.

The work presented in this thesis was carried out from January 2008 to July 2011 under supervision of Associate Professor Lone Brøndsted and Postdoctoral Fellow Marianne Thorup Cohn. The majority of the work was carried out at the Department of Disease Biology at the Faculty of Life Sciences in Frederiksberg in Denmark. Some of the work was performed at London School of Hygiene and Tropical Medicine in London in the United Kingdom. The PhD study was financed by the European Union funded Integrated Project BIOTRACER.

Frederiksberg, 23<sup>rd</sup> of September 2011

Małgorzata Ligowska

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Finally, I thank my boyfriend Robert for his endless support and encouragement during the whole duration of the project and also my parents and my brother for their great interest, encouragement and support at all times.



## Summary

The majority of bacterial gastrointestinal infection cases in developed countries are caused by the human pathogen *Campylobacter jejuni* and poultry meat is considered the main food source responsible for these infections. As a pathogen, *C. jejuni* is exposed to frequent fluctuations in the nutrient availability, temperature, as well as shifting oxygen concentration. Changes in these conditions are likely to cause stress to the bacterium. Being a microaerophile, *C. jejuni* is particularly vulnerable to atmospheric oxygen concentrations and the resulting accumulation of Reactive Oxygen Species (ROS) can cause great damage to the major cellular components. *C. jejuni* has adapted to these oxidative stress conditions by activating an array of proteins that can prevent deleterious effects caused by ROS accumulation.

Among these proteins, Dps protein (DNA-binding protein from starved cells) plays an important role during oxidative stress. It sequesters free iron from the medium, thereby preventing the Fenton reaction to occur, which would otherwise lead to formation of toxic hydroxyl radicals. In this thesis, a novel phenotype associated with this protein was described. *C. jejuni* cells from exponential phase lacking the Dps protein were more sensitive to peroxide stress than cells in stationary phase. This discovery has led to further exploration of growth phase differences in response to oxidative stress. It was revealed that the expression from the *dps* gene is controlled at either post-transcriptional or post-translational level and that this regulation occurs to a greater extent in the stationary phase. Furthermore, the presence of iron was shown to have an influence on *dps* transcript level induction that differed between the growth phases. Iron was required for the oxidative stress-mediated induction of the *dps* transcript level in exponential phase cells, while in stationary phase cells the presence of iron during oxidative stress contributed to a decrease in the *dps* transcript level. The role of two iron-dependent repressors, PerR and Fur, known to regulate Dps, was also shown to differ between growth phases. In the exponential phase both PerR and Fur acted as repressors of *dps* transcription, whereas in the stationary phase Fur acted still as a repressor, while PerR became an activator of *dps* in the absence of Fur. Transcription of certain other oxidative stress genes (*katA* encoding catalase and *ahpC* encoding alkyl hydroperoxidase) differed among growth phases and expression of another oxidative stress gene, *sodB* encoding superoxide dismutase, was repressed in the absence of Dps in the stationary phase culture.

*C. jejuni* has also adapted to the environment in chicken meat juice, in which it can survive for up to 30 days at low temperatures (4°C), which corresponds to conditions of storage of poultry meat products and thereby increases the risk of developing a disease by a consumer. A microarray study was performed, in which gene expression of *C. jejuni* kept in laboratory medium at low

temperature was compared with gene expression of *C. jejuni* kept in chicken meat juice. Careful adjustment of the experimental settings allowed for identification of only a few genes whose expression was changed specifically in response to chicken meat environment and therefore contributed to the adaptation of *C. jejuni* to this environment. Among those genes that had their transcript increased in chicken meat juice were genes involved in iron and cryoprotectants uptake, metabolism and quorum sensing and glycosylation. Furthermore, comparison of survival of a  $\Delta luxS$  mutant with the wild type strain in chicken meat juice revealed that presence of LuxS is required for long term survival of *C. jejuni* in this environment at low temperature.

To sum up, the work presented in this PhD thesis focused on adaptation of *C. jejuni* to oxidative stress and poultry meat environment. It was shown that oxidative stress response involving Dps protein and certain other known oxidative stress genes was growth phase dependent and interesting differences were revealed pointing to post-transcriptional or post-translational regulation. The effect of chicken meat environment on gene expression in *C. jejuni* was investigated and several genes specifically contributing to the adaptation to this environment were identified.

## Dansk resumé

De fleste tilfælde af bakterielle mave-tarm infektioner i den udviklede verden er forårsaget af den humane patogene bakterie *Campylobacter jejuni*, og fjerkrækød betragtes som den vigtigste fødevarekilde til disse infektioner. *C. jejuni* er udsat for hyppige udsving i temperatur, tilgængeligheden af næringsstoffer og iltkoncentrationer og ændringer i disse forhold kan forårsage stress hos bakterien. *C. jejuni* er en mikroaerofil bakterie og er derved særligt sårbar over for atmosfæriske iltkoncentrationer og den resulterende ophobning af reaktive iltradikaler, som forårsager store skader på de vigtigste cellulære komponenter. Når *C. jejuni* udsættes for oxidativ stress, aktiverer bakterien en række proteiner der forhindrer de skadelige følgevirkninger af iltradikalernes ophobning.

Et af disse proteiner er Dps, som spiller en vigtig rolle under oxidativt stress. Ved at binde frit jern i mediet, forhindrer Dps Fenton-reaktionen i at forløbe, hvilket ellers ville føre til dannelse af giftige hydroxylradikaler. I denne afhandling beskrives en ny fænotype forbundet med Dps proteinet, i hvilken eksponentielt voksende *C. jejuni* celler uden Dps er mere følsomme overfor peroxidstress end celler i stationær fase. Denne opdagelse førte til yderligere udforskning af forskelle mellem vækstfaser i respons på oxidativt stress. Det viste sig at ekspressionen af *dps* genet er kontrolleret på enten post-transkriptionelt eller post-translationelt niveau, og at denne regulering sker i større omfang i stationær fase. Endvidere viste tilstedeværelsen af jern sig at have indflydelse på transkriptionsniveauet af *dps*, som varierede med vækstfasen. Jern var nødvendigt for den oxidativt stress-medierede induktion af *dps* transkriptionsniveauet i eksponentiel fase, mens tilstedeværelse af jern i stationær fase under oxidativt stress bidrog til et fald i niveauet af *dps* transkription. De to jern-afhængige repressorer, PerR og Fur, der er kendt for at regulere Dps, viste sig også at have forskellige roller afhængig af vækstfasen. I den eksponentielle fase fungerede både PerR og Fur som repressorer af *dps* transskriptionen, mens PerR i den stationære fase aktiverede *dps* i fravær af Fur. Transskription af visse andre oxidativt stress-relaterede gener (*katA* og *ahpC*) varierede også afhængig af vækstfasen og ekspressionen af et anden oxidativt stress-relateret gen, *sodB*, blev undertrykt i fravær af Dps i stationær fase.

*C. jejuni* kan overleve i op til 30 dage i kyllingekødssaft ved lave temperaturer (4°C), hvilket svarer til vilkårene for opbevaring af fjerkrækødprodukter, hvorved risikoen øges for udvikling af sygdom hos forbrugeren. En microarray undersøgelse blev udført, hvor genekspression hos *C. jejuni* i laboratorie-medium ved lav temperatur blev sammenlignet med genekspression hos *C. jejuni* i kyllingekødssaft. Omhyggelig justering af de eksperimentelle forhold gjorde det muligt at identificere ganske få gener, hvis udtryk blev ændret specifikt som reaktion på kyllingekød-miljøet

og dermed bidrog til tilpasningen af *C. jejuni* til dette miljø. Blandt de gener, hvis ekspression steg i kyllingekødsaft var *cfbpC*, *luxS*, *cj1323* og *cdtA*. Endvidere viste en undersøgelse af overlevelsen i kyllingekødsaft, at tilstedeværelsen af proteinet LuxS er nødvendig for langsigtet overlevelse af *C. jejuni* i dette miljø ved lav temperatur.

For at opsummere, har det arbejde, der præsenteres i denne ph.d.-afhandling været fokuseret på tilpasning af *C. jejuni* til oxidativt stress og fjerkrækød-miljø. Det blev vist, at det oxidative stressrespons, som involverer Dps og visse andre kendte oxidativt stress-relaterede gener er væksthase-afhængigt, og interessante forskelle som peger på post-transkriptionel eller post-translationel regulering blev identificeret. Effekten af kyllingekød-miljøet på genekspression i *C. jejuni* blev undersøgt, og flere gener som specifikt bidrager til tilpasning til dette miljø blev identificeret.

# **Chapter 1**

## **Introduction**

# 1. Introduction

## 1.1 *Campylobacter jejuni* physiology

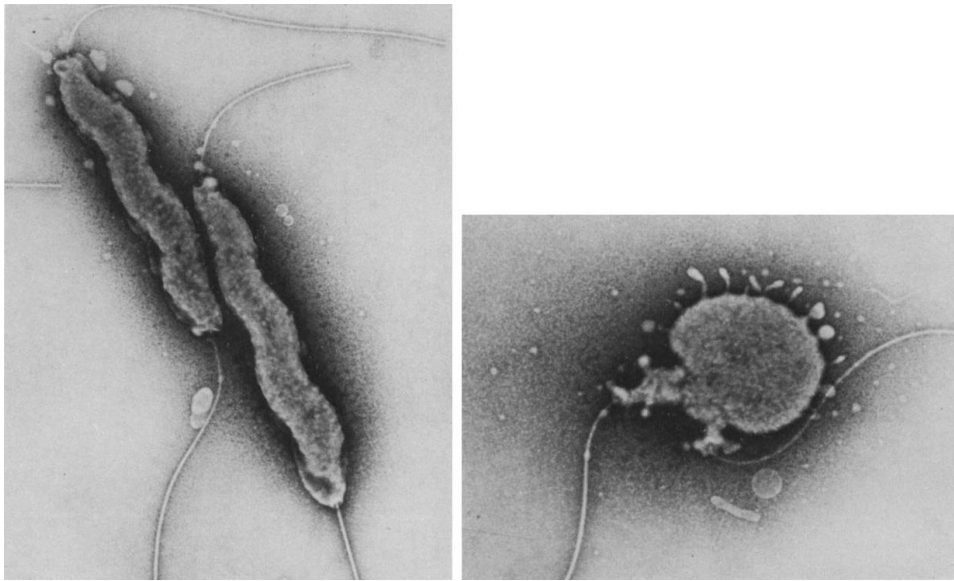
### 1.1.1 The discovery

In the year 1919 a paper was published describing in detail morphological, cultural and biological characteristics of a microorganism, known to cause infectious abortion in cattle, which the authors named spirillum, owing it to its shape, (Smith and Taylor 1919). The authors observed that “In films and in hanging drop preparations from fetal fluids and cultures therefrom, the spirilla appear as fine wavy or sinuous lines of various lengths. The smallest forms appear as minute curved S-shaped lines; the longest may stretch nearly across the field of the microscope. (...)”. They termed the microorganism *Vibrio fetus*. Many years later, it became apparent that this bacterium differs from the *Vibrio* genus in several aspects and a new genus, *Campylobacter*, was suggested (Sebald and Veron 1963). The main differences were reported in the GC content, which was lower in *Campylobacter* (29-36 mol %) than in *Vibrio* (40-53 mol %) and in an inability of the newly assigned *Campylobacter* genus to ferment carbohydrates, in contrast to *Vibrio* (Veron and Chatelain 1973). *Campylobacter jejuni* today is commonly known as the pathogen causing the largest number of diarrhea in humans (Allos 2001). The first time a connection was made between a “related vibrio” and human diarrhea was in 1973 when Butzler et al. examined 800 stools from infants, children and 100 adults with diarrhea and compared them with stools from 1000 children without diarrhea. The “related vibrios” were recovered from 13 stools from patients without diarrhea, indicating a carrier state, as well as from 52 children stools and 4 adult stools from patients with diarrhea, pointing to the link between the microorganisms and the disease (Butzler et al. 1973).

Today the *Campylobacter* genus consists of 18 species and 6 subspecies classified by comparison of 16S rRNA gene sequences (Humphrey et al. 2007; On 2001). Among these, *Campylobacter jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei*, *Campylobacter coli*, *Campylobacter lari*, *Campylobacter upsaliensis* and *Campylobacter helveticus* are most commonly isolated from human and animal diarrheal specimens (On 2001). The *Campylobacter* genus belongs to the epsilon subdivision of the *Proteobacteria* classified based on characterization of 23S rRNA sequence (Trust et al. 1994). Other members of this subdivision include *Arcobacter*, *Helicobacter* and *Wolinella* genera. All the work described in this thesis focused on *Campylobacter jejuni* subsp. *jejuni* with the following lineage: Superkingdom: Bacteria, Phylum: Proteobacteria, Subphylum:  $\delta/\epsilon$  subdivisions, Class:  $\epsilon$ -proteobacteria, Order: Campylobacterales, Family: Campylobacteraceae, Genus: *Campylobacter*, Species: *Campylobacter jejuni*.

### 1.1.2 Morphology and physiology

*Campylobacter jejuni* is a Gram-negative, spiral-shaped motile bacterium with width ranging from 0.2 to 0.8  $\mu\text{m}$  and length from 0.5 to 5  $\mu\text{m}$  (Nachamkin et al. 2008). They have a polar flagellum at each end of a cell. In late stationary phase cultures a coccoid cell form predominates (Figure 1-1).



**Figure 1-1. *Campylobacter jejuni* spiral (left) and coccoid (right) form. (Peard 1979)**

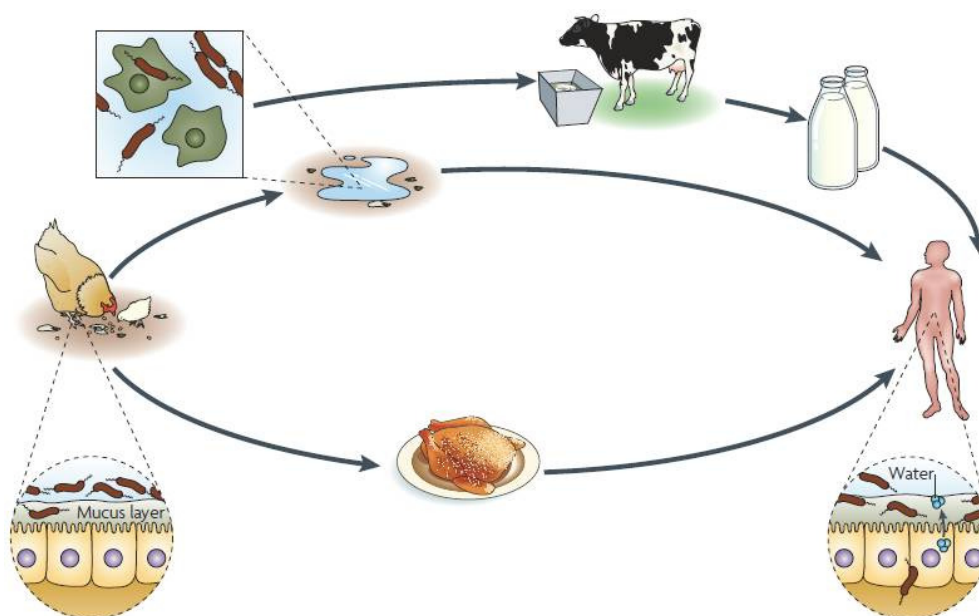
*Campylobacter* spp. is characteristic among other pathogens by having fastidious growth requirements. They have a rather narrow range of growth temperatures, between 30°C and 45°C (Hazeleger et al. 1998; Park 2002), with an optimal growth temperature being 42°C (Park 2002). In addition to that, they are considered capnophilic and microaerophilic, requiring an atmosphere with carbon dioxide and containing low oxygen concentrations. The optimal atmosphere for cultivation of *C. jejuni* contains 5%-10% O<sub>2</sub> and 1-10% CO<sub>2</sub> (Bolton and Coates 1983; Luechtefeld et al. 1982).

### 1.1.3 Lifestyle as a pathogen

*Campylobacter* genus is the main cause of bacterial foodborne disease in the developed world. In the United States, according to the FoodNet surveillance network of the Centers of Disease Control and Prevention (CDC), it infects over 2.4 million people every year, which is 0.8% of total population. According to the World Health Organization (WHO) the numbers are similar for

Western Europe, where 1% population is estimated to get infected with *Campylobacter* every year. Even though the numbers are high, a characteristic feature of *C. jejuni* infection in humans is that outbreaks are rarely reported and if they occur, they are of limited extent (Gardner et al. 2011; Heuvelink et al. 2009; Karagiannis et al. 2010; Stuart et al. 2010).

Despite very specific growth requirements, the *Campylobacter* genus is widely spread in the environment. Poultry seems to be the main reservoir of *Campylobacter jejuni* that colonizes the gastrointestinal tract of chickens and significant numbers are isolated predominantly from the large intestine, cecum and cloaca (Beery et al. 1988). *C. jejuni* has also been isolated from wild birds such as migratory ducks (73%), Canada geese (5%) and sandhill crane (81%) suggesting a potential role of wild birds in spreading this pathogen at large distances (Pacha et al. 1988). *C. jejuni* was also isolated from companion animals such as cats and dogs (Blaser et al. 1980). Water-associated outbreaks of infection caused by *C. jejuni* have also been reported (Alary and Nadeau 1990; Hänninen et al. 2003; Richardson et al. 2007). An overview of possible sources of *C. jejuni* and transmission routes that can eventually lead to developing infection in humans is illustrated in (Figure 1-2).



**Figure 1-2. The sources of *Campylobacter jejuni* (Young et al. 2007).** The main reservoir of *C. jejuni* in the environment is considered to be poultry. *C. jejuni* is efficient at colonizing chicken gastrointestinal tract, reaching high numbers, and can be then passed inside a chicken flock through fecal-oral route. When *C. jejuni* enters a water supply, it can use protozoans such as amoebae as a vector or likely form biofilms that help it survive for longer periods. Humans can get infected through consumption of contaminated animal products such as poultry meat or unpasteurized milk. Once *C. jejuni* reaches human gastrointestinal tract, it invades the epithelial layer and causes inflammation and diarrhea.



A Scottish study compared genotypes of 5674 clinical isolates of *C. jejuni* with 5837 isolates from potential human infectious sources which allowed the attribution of infections to the most probable source. It was confirmed to be the chicken meat by both models applied in that study (Sheppard et al. 2009). Other sources of human infection included mostly sheep and cattle, as well as wild birds, environment, swine and turkey sources, where the last four were of very low probability.

*C. jejuni* is characterized by a very low infectious dose (500 to 800 bacteria) (Black et al. 1988; Robinson 1981). Once *C. jejuni* reaches a human host, a disease called campylobacteriosis may develop within 2 to 5 days (Young et al. 2007). Typical disease symptoms include watery or bloody diarrhea, abdominal pain and fever. Even though the disease is usually self-limiting, usually lasting for no longer than a week, rare but serious complications may occur in up to weeks following the infection. These complications include, among others, reactive arthritis and the Guillain-Barré syndrome (GBS), an acute disorder affecting the peripheral nervous system (Nachamkin 2002).

## **1.2 Campylobacter in the food chain**

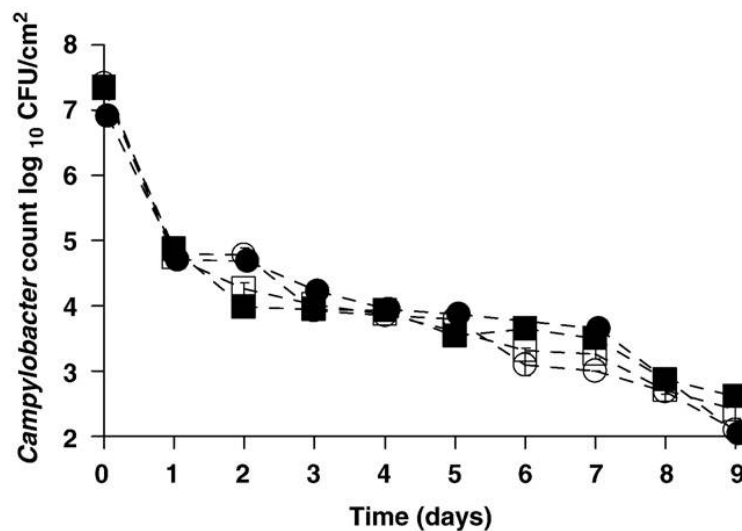
In poultry as in many other animals, *C. jejuni* is a commensal, only rarely, or not at all, causing a disease (Blaser et al. 1980). Poultry flocks are infected with *C. jejuni*, presumably from the environment, within the first weeks, and become colonized at high levels of  $10^6 - 10^7$  CFU/g in the cecum (Corry and Atabay 2001). Contamination of the chicken meat with bacteria from the intestines can occur during routine procedures at the slaughter house such as defeathering, evisceration or carcass chilling. Those were found to be responsible for contamination of the meat with *Campylobacter* (Bashor et al. 2004). Due to the low infection dose (Black et al. 1988; Robinson 1981) consumers may easily come into contact with *Campylobacter* present on a meat product and ingest the bacteria by accident through improper meat handling in the kitchen, cross-contamination of products that are not heat treated or by consuming undercooked poultry. *C. jejuni* has developed different ways of adapting to a variety of environments and conditions that it encounters on the way from farm to fork.

### **1.2.1 Survival at low temperatures in meat environment**

It is known that *C. jejuni* cannot multiply on a meat product stored, for example, in a fridge, because of its narrow temperature range for growth (Hazeleger et al. 1998) and the lack of microaerobic atmosphere. However, several factors ensure a long term survival of *C. jejuni* in meat products.

Purchased chicken meat is typically stored at either 4°C or -20°C. Several studies have investigated how *C. jejuni* responds to these temperatures and their main findings are reported below.

One interesting study investigated the effect of freezing (-20°C) temperatures on survival of *C. jejuni* and *C. coli* on chicken skin (El-Shibiny et al. 2009).

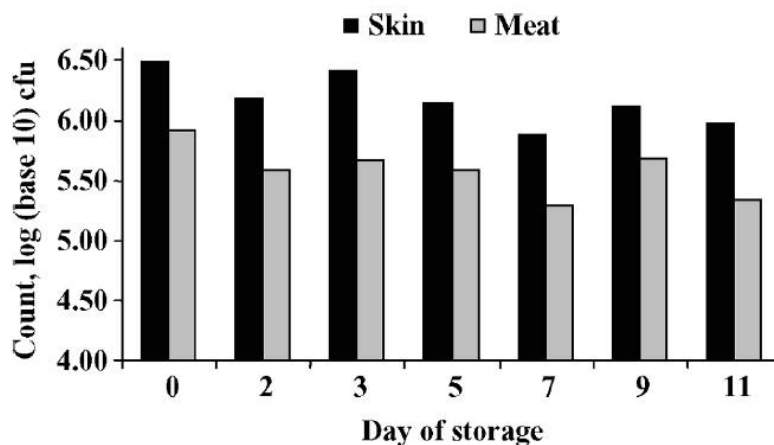


**Figure 1-3. Survival of *C. jejuni* and *C. coli* inoculated on to chicken skin and chilled in a domestic freezer to -20°C (El-Shibiny et al. 2009).** *C. jejuni* isolates from a UK organic chicken farm (circles), *C. coli* isolates (squares), antibiotic resistant.

As presented in Figure 1-3, the effect of freezing has a much more dramatic effect in reducing the numbers of *C. jejuni* and *C. coli*, as a reduction between 2.2 log<sub>10</sub> and 2.6 log<sub>10</sub> CFU/cm<sup>2</sup> is observed during the first 24 hours. Nevertheless, after the large initial reduction, the two strains remain on the skin in numbers well above the infectious dose (Black et al. 1988; Robinson 1981) for approximately 8 days. This study indicated that the chicken skin environment is favourable for *C. jejuni* to survive at freezing temperatures. In yet another study it was, however, pointed out that the composition of the medium around the bacteria, rather than the chicken skin surface was the factor that contributed the most to survival of *C. jejuni* at freezing temperatures (Birk et al. 2006). In a comparison of two food model systems, a liquid and a skin model, *C. jejuni* survived better at -18°C when it was suspended in chicken juice than when it was inoculated on chicken skin and then frozen.

A study by Davis and Conner (2007) compared the ability of *C. jejuni* to survive on meat products with and without skin at refrigeration temperatures (4°C) and -3°C which corresponded to surface ice crusting temperature (Davis and Conner 2007). Bacteria were artificially introduced

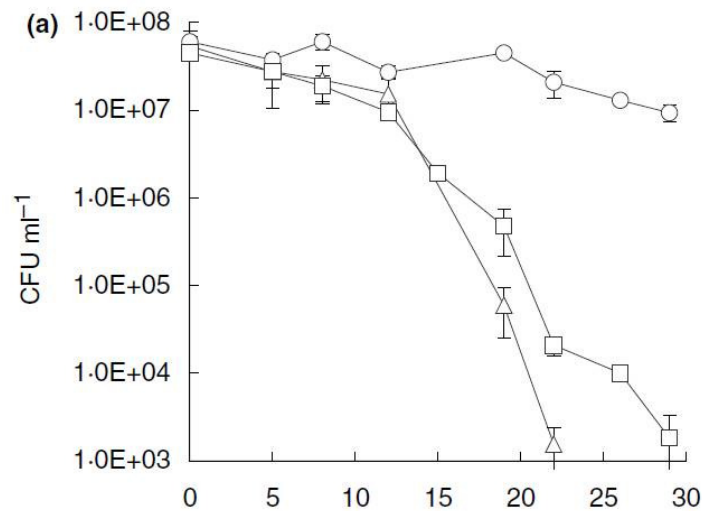
onto the skin and meat and survival determined in the course of 11 days. From the results it was evident that at these temperatures the number of *C. jejuni* cells was not significantly reduced within 11 days on meat or skin (Figure 1-4).



**Figure 1-4. Comparison of average populations of *C. jejuni* on inoculated poultry skin vs. meat at different days of storage at all temperatures (Davis and Conner 2007).**

The inoculation levels of *C. jejuni* used in the above mentioned study ( $\sim 5.0 \times 10^5$  CFU/ml) corresponded to those found in fecal material from poultry and therefore represented a realistic situation and a risk that might be encountered by a consumer.

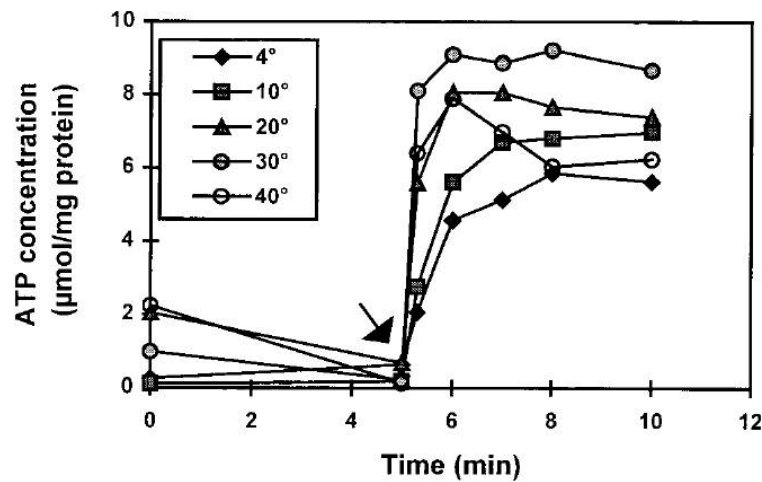
In a study published by Birk et al. (2004), survival of *C. jejuni* in chicken juice was compared to survival in a laboratory medium, BHI, at low temperatures (Birk et al. 2004). Here, a remarkably better survival of *C. jejuni* at low temperatures was reported when bacteria were in chicken juice at 5°C. An almost unaltered survival was seen for 30 days in chicken juice compared to BHI, where a decrease in survival was observed already after 12 days (Figure 1-5).



**Figure 1-5. Survival curves of *C. jejuni* 11168 incubated at 5°C.** Average CFU/ml after incubation in BHI broth (triangles), chicken juice (circles), or BHI broth supplemented with 5% calf blood (squares) in a microaerobic atmosphere (Birk et al. 2004).

Similarly, *C. jejuni* was also more tolerant to heat stress when present in chicken juice compared to BHI. This study provided strong evidence that chicken meat environment, whether it is meat, skin or “juice”, provides an advantage to *C. jejuni* and allows increased survival even under stress, such as heat or cold shock.

It is, in fact, puzzling how *C. jejuni* is able to survive at such low temperatures for many days, given the fact that it has not been described that *C. jejuni* contains any of the known cold shock proteins such as CspA (Parkhill et al. 2000), which in *E. coli* plays a major role during cold shock, acting as RNA chaperone (Yamanaka et al. 1998). Interestingly, it has been shown that at low temperatures *C. jejuni* exhibits physiological activity in the form of oxygen consumption, catalase activity, protein synthesis and ATP generation (Figure 1-6).



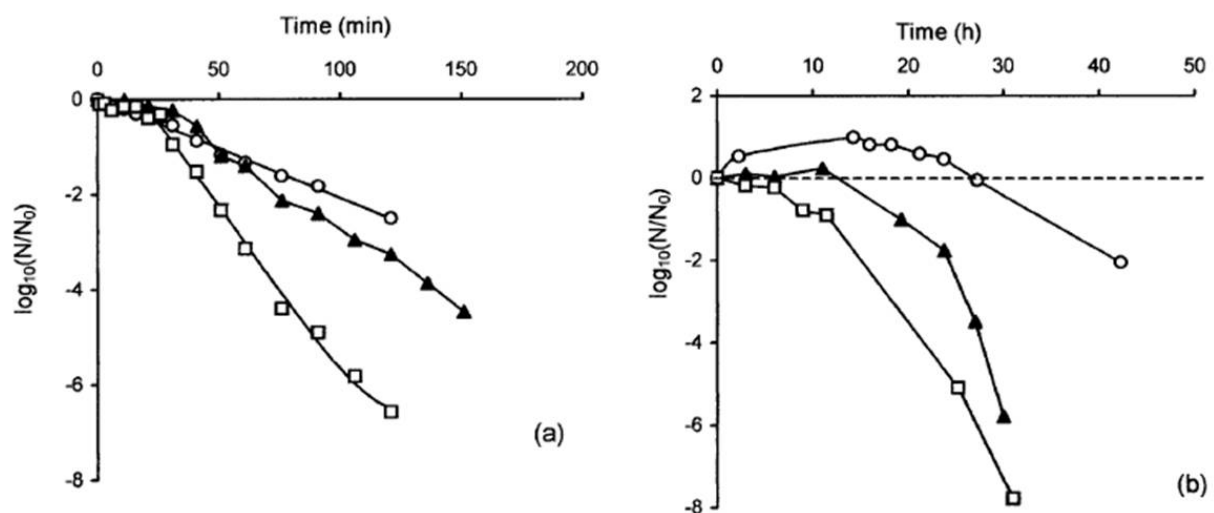
**Figure 1-6. Effect of temperature on the ATP production of *C. jejuni* ATCC 33560 (Hazeleger et al. 1998).**

These results indicate that *C. jejuni* is in fact adapted to survival at low temperatures, but presumably using mechanisms different from those previously identified in other bacteria. In order to address the question of how *C. jejuni* can adapt to low temperatures, Stintzi and Whitworth (2003) conducted a genome-wide transcriptome analysis of *C. jejuni* that was submitted to temperature decreases from 42°C to 37°C, 32°C, 10°C or 4°C (Stintzi and Whitworth 2003). The result was that an impressive 13% of the genome was significantly changed by cold shock and that this response was related to oxidative stress, as genes such as *sodB* and Cj0358 (putative cytochrome C551 peroxidase) were upregulated. Both genes are responsible for removal of free radicals and the authors suspect that these would be abundant during cold shock, since *C. jejuni* cannot grow at 30°C and yet it continues to respire. This valuable study has also identified potential mechanisms which could contribute to the adaptation to low temperatures. These included acquisition or biosynthesis of cryoprotectant molecules, maintenance of translation initiation and efficiency, alteration of the membrane lipid composition and upregulation of several two-component systems, which could be helpful in sensing the environment changes and eliciting a proper response.

### 1.2.2 Heat sensitivity and growth phase differences

*C. jejuni* was also shown to be sensitive to heat stress and Moore and Madden (2000) have concluded that any thermal treatment meant to eradicate either *Salmonella* or *Listeria* will be sufficient to kill *Campylobacter* as well (Moore and Madden 2000). In that study, an interesting behavior of *C. coli* was reported while examining death kinetics of this organism. In the range of

temperatures from 48.8°C to 55.1°C the death rate was logarithmic, which indicates first-order death kinetics. However, above temperatures of 56°C a deviation to non-logarithmic reduction of cells was observed and this suggested the existence of heat-resistant subpopulation within the parent population. This finding is extremely important to take into account while preparing foods that require only mild heat treatment, as it might not be sufficient to kill all subpopulations of *Campylobacter*. Another unusual response of *C. jejuni* to heat stress was observed in the study by Kelly et al. (2001), where resistance to mild heat treatment (50°C) was compared between *C. jejuni* 11351 in exponential phase, early stationary phase (24h) and late exponential phase (48h) (Kelly et al. 2001). In this study the exponential phase cells were found to be the most resistant to this heat treatment (Figure 1-7).

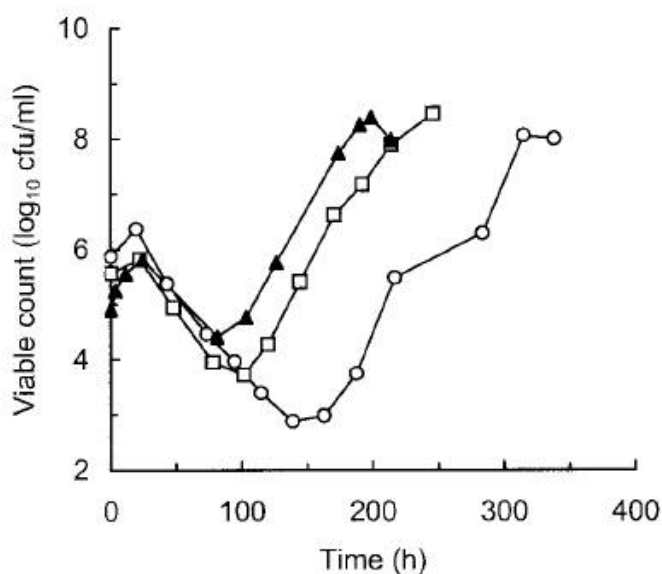


**Figure 1-7. Effect of growth phase on resistance of *C. jejuni* 11351 to heat and aeration.** Cells were grown to exponential phase (circles), early stationary phase (24h)(triangles) and late stationary phase (48h)(squares). Samples were heated to 50°C (a) or diluted in prewarmed PBS prior to aeration at 37°C and 90 rpm. Modified from Kelly et al. 2001.

This finding was different, but not surprising, compared to what has been found for other Gram negative bacteria such as *E. coli*, where stationary phase cultures are more resistant to stress (Rees et al. 1995). It is known that *C. jejuni* does not have a RpoS homologue (Parkhill et al. 2000), which in *E. coli* is induced in stationary growth phase and governs physiological changes in the cell that make it more resistant to different types of stress. In this light, the fact that stationary phase cells in *C. jejuni* are more sensitive to stress therefore nicely corresponds to the lack of RpoS homologue in this bacterium. At the same time, this study indicated that there could be interesting

differences in the behavior of *C. jejuni* between the growth phases that might differ from what is common in other microorganisms.

In fact, an interesting observation was made by Kelly et al. (2001) while investigating heat resistance of *C. jejuni* during extended stationary phase. Samples were taken out at regular intervals during growth and submitted to heat challenge at 50°C for 75 minutes. As expected, the exponential phase cells showed increased heat resistance and as the cells entered stationary phase, the resistance decreased. After 50 hours of incubation, surviving cells were below the detection limit, but after 72 hours the heat resistance has unexpectedly increased again. The authors attributed this phenomenon to the presence of bacteria in the population that were more resistant to heat and eventually took over the growth. The group has published a study two years later, examining this “phoenix” phenomenon, as they called it, in more detail. This time *C. jejuni* cultivated at 39°C was transferred to a fresh medium and grown at 30°C (Kelly et al. 2003). A similar growth pattern appeared, where there was an initial increase in the growth followed by a decline, and then the growth was initiated again, reaching higher numbers than the initial inoculum (Figure 1-8).



**Figure 1-8. Growth of *C. jejuni* NCTC 11351 grown at 39°C and inoculated into fresh medium at temperatures near the minimum for growth (Kelly et al. 2003).** Triangles: exponential phase inoculum, 30°C; squares: stationary phase inoculum, 30°C; circles: stationary phase inoculum, 29°C.

This behavior was interpreted as a result of combined effect of the growth of the cells at minimum growth temperature and during oxidative stress, resulting in loss of viability and finally emergence of a spontaneous mutant that took over the culture. The authors suggested that the

presence of hypervariable sequences (Parkhill et al. 2000), abundant in this bacterium, might be the reason for the genetic variability, allowing spontaneous mutants to arise and successfully take over the culture. Thus, this might be the way by which *C. jejuni* compensates for the lack of RpoS and uses a different survival strategy.

*C. jejuni* is certainly an enigmatic pathogen that seems to have its unique ways of adapting to the different environments in which it can be found. Its successful strategy seems to combine low infectious dose, potential for creating genetic variability and a unique adaptation to cold shock, mechanisms of which are yet to be unraveled.

### 1.3 Stress response

Stress conditions seem to be an inevitable part of every pathogen's life. These microorganisms are very specialized and well adapted to their hosts, in which they can avoid or trick the immune system and successfully establish a disease. However, these microorganisms meet many challenges on the way to and within the host, and must be able to survive and grow in a variety of environments. Encountered stress conditions might include heat, oxidative stress, cold shock, desiccation, varying pH, starvation or osmotic stress. In order to cope with these conditions, *C. jejuni* has developed an array of specialized mechanisms in response to external factors. In this chapter I will mostly focus on oxidative stress and iron homeostasis in the cell.

#### 1.3.1 What is oxidative stress?

All organisms that require oxygen for respiration or other biochemical reactions, utilize the molecular oxygen ( $O_2$ ) to form  $H_2O$  (Miller and Britigan 1997). However, during these reactions oxygen can be sequentially reduced to a variety of toxic oxygen intermediates called Reactive Oxygen Species (ROS). These include superoxide anion radical ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $OH^\bullet$ ) (Cabiscol et al. 2000) and the reduction is depicted in Figure 1-9.



**Figure 1-9. Schematic presentation of reduction of molecular oxygen to water and the intermediates of this reaction.** From left to right: molecular oxygen, superoxide anion, hydrogen peroxide, hydroxyl radical and water.

Since they are a part of the normal aerobic metabolism, and also short-lived, most of the time they do not pose a threat to a cell. However, when too much ROS accumulates in a bacterium,



the concentrations are beyond cell's defensive capacity and they cause oxidative stress. This can happen as a result of presence of various environmental factors such as near UV radiation (NUV), or certain redox-cycling antibiotics released by plants and other microorganisms in order to eliminate competing organisms (Storz and Imlay 1999). Additionally, cells of the immune system, such as phagocytes, generate ROS and direct them towards the encapsulated bacteria (Lambeth 2004).

### **1.3.2 Damage caused by oxidative stress**

Once ROS concentration exceeds the "normal" concentration for a given cell, the oxidative damage starts to be visible. It seems that some of the individual ROS have their preferred targets in the bacterial cell. Superoxide, which is considered a moderately reactive compound, and for this reason can move longer distances before reaching its target, affects bacterial enzymes, especially those involved in biosynthesis of branched amino acids (Haas and Goebel 1992; Kuo et al. 1987). Superoxide requires cellular anion channels to be able to diffuse inside a cell (Roos et al. 1984). Hydrogen peroxide, on the other hand, can readily diffuse through membranes and is also more reactive than superoxide (Miller and Britigan 1997), being able to oxidize and damage more cellular components, such as cell membranes, enzymes, DNA and it can also inhibit membrane transport processes (Weiss 1986). Hydroxyl radical is considered to be the most reactive ROS although it is difficult to assess whether the damage in a cell is caused by the precursors  $\cdot\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  or by hydroxyl radical alone (Miller and Britigan 1997). The targets of hydroxyl radicals include proteins, DNA and lipids (Du and Gebicki 2004). The presence of iron in a cell has a big impact on the level of oxidative stress as well as targets for oxidation and I will touch upon this subject in the next sections.

The mode of action of ROS might be similar in many different organisms, prokaryotic and eukaryotic alike, but what causes stress is certainly individual for each organism and dependent on its lifestyle. One could say that for *C. jejuni* the situation is quite unique in that, being a microaerophilic bacterium it requires oxygen for growth, but at very low levels and at the same time it is constantly exposed to oxidative stress whenever it is inside the host or outside, in the environment.

### **1.3.3 Defense against oxidative stress**

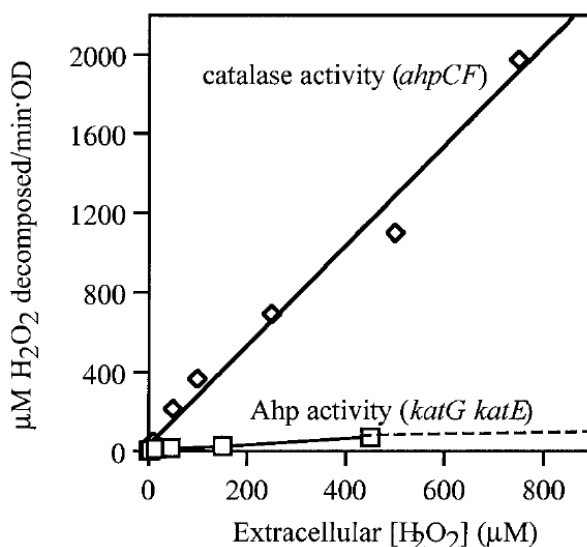
*C. jejuni*, as well as other pathogens, has evolved mechanisms that protect them from the destructive effects of oxidative stress. The first intermediate in the process of oxygen reduction is the superoxide ( $\cdot\text{O}_2^-$ ) and accordingly, the first line of defense against oxidative stress in *C. jejuni* is

thought to be superoxide dismutase (SOD) (Purdy and Park 1994) which acts by catalyzing the conversion of superoxide to hydrogen peroxide and molecular oxygen (Purdy et al. 1999). SOD enzymes require a metal cofactor for catalysis and so far, four general types of SOD have been described in organisms: containing iron cofactors (FeSOD or SodB), manganese cofactors (MnSOD or SodA) (Hopkin et al. 1992), containing both copper and zinc cofactors (CuZnSOD or SodC) (Steinman 1993) and nickel cofactor (NiSOD) (Wuerges et al. 2004). Whereas *E. coli* contains both FeSOD and MnSOD (Gregory et al. 1973), *C. jejuni* contains only one, cofactored with iron (SodB) (Pesci et al. 1994). Studies on a *C. jejuni* *sodB* mutant revealed that even though it was able to grow in a liquid culture in aerobic atmosphere just as well as the wild type, when an additional source of superoxides, methyl viologen, was added, the mutant could not grow (Purdy et al. 1999). Furthermore, the *sodB* mutant's survival in aerobic atmosphere and in food models such as skimmed milk and chicken skin was severely impaired and the mutant was less efficient at colonizing 1-day old chicks than the wild type. Another study has focused on investigating the role of *sodB* during freeze-thaw stress in *C. coli* (Stead and Park 2000), as it has been implicated before that cell injury that occurs during this process is due to a combination of several factors, among others, oxidative burst that can happen during thawing (Park 2002). Indeed, the *sodB* mutant was shown to be more sensitive to freezing and thawing than the wild type of *C. coli* in this case.

Another enzyme playing an important role during protection from oxidative stress is catalase, which role is to reduce  $H_2O_2$  to water and oxygen. Several microorganisms are known to contain more than one of the three known types of catalases: monofunctional, bi-functional catalase-peroxidases and manganese-containing catalases (Chelikani et al. 2004), however *Campylobacter* spp. contains only one, KatA. A *kata* mutant of *C. coli* was shown to be more sensitive to killing by  $H_2O_2$  than the wild type (Grant and Park 1995) and catalase in *C. jejuni* was shown to contribute to cell growth in macrophages, but not in epithelial cells (Day et al. 2000). In contrast to SodB, KatA was not necessary for colonization of the chicken gut in *C. coli* (Purdy et al. 1999) and not involved in freeze-thaw stress (Stead and Park 2000). Interestingly, addition of pyruvate to the growth medium led to reduced KatA activity and allowed *C. jejuni* to grow aerobically (Verhoeff-Bakkenes et al. 2008). The authors concluded that the reason for it is that pyruvate can eliminate  $H_2O_2$  from the medium just as efficiently as the enzymes such as catalase.

In addition to catalase, alkyl hydroperoxidase (AhpC) is also capable of scavenging hydrogen peroxide. It belongs to a group of antioxidant enzymes called peroxiredoxins that act by converting hydrogen peroxide as well as organic hydroperoxides to water and a corresponding alcohol (Wood et al. 2003). An interesting study in *E. coli* revealed that the activity of catalase and

alkyl hydroperoxidase is dependent on  $\text{H}_2\text{O}_2$  concentration with alkyl hydroperoxidase scavenging  $\text{H}_2\text{O}_2$  optimally at very low concentrations (5  $\mu\text{M}$ ) and catalase taking over that role when alkyl hydroperoxidase activity became saturated at 20  $\mu\text{M}$  (Figure 1-10) (Seaver and Imlay 2001).



**Figure 1-10. Dependence of scavenging rate on  $\text{H}_2\text{O}_2$  concentration.** Rates of  $\text{H}_2\text{O}_2$  decomposition were measured in dilute suspensions of  $\Delta\text{ahpCF}$  mutant (diamonds) and  $\Delta\text{katG } \Delta\text{katE}$  mutant (squares). Rates were normalized to a value of OD 1.0. (modified from Seaver and Imlay 2001)

So far it is not known whether a similar dependency exists between *C. jejuni* AhpC and KatA. Studies of a *C. jejuni* *ahpC* mutant revealed that cells lacking this protein were much more sensitive to cumene hydroperoxide (CHP) than the wild type cells both in a disk inhibition assay as well as in liquid cultures (Baillon et al. 1999). The resistance to hydrogen peroxide, however, was not affected, indicating specificity of the AhpC protein towards CHP. Additionally, the *ahpC* mutant was much more sensitive to atmospheric air conditions than the wild type.

*C. jejuni* contains another peroxiredoxin named thiol peroxidase (Tpx). The homologues of this enzyme in other bacteria such as *E. coli* or *H. pylori* are known to use various peroxides as substrates (Cha et al. 2004; Comtois et al. 2003). In *C. jejuni* however, Tpx was shown to have its substrate specificity limited to hydrogen peroxide (Atack et al. 2008). At the same time, the protein was induced in the wild type cells growing at aerobic conditions compared to microaerobic conditions, but not when the cells were grown with hydrogen peroxide.

#### 1.3.4 Role of iron

Virtually all life forms are dependent on iron, with the remarkable known exceptions of *Lactobacillus plantarum* among bacteria (Archibald 1983) and *Borrelia burgdorferi* among parasites (Posey and Gherardini 2000) which have evolved to substitute iron with divalent manganese ion. Iron is crucial for a number of biological processes, among others cellular respiration, oxygen transport, gene regulation, DNA biosynthesis and many others (Andrews et al. 2003; Madigan and Martinko 2006). Iron, being the fourth most abundant element in the Earth's crust (Litwin and Calderwood 1993), can occur in the environment in several forms, for example as FeS, Fe(OH)<sub>3</sub> and many other iron salts (Madigan and Martinko 2006). Depending on the availability of oxygen, it can occur in two redox states. The reduced Fe<sup>2+</sup> state (ferrous iron) is more common in anoxic conditions and soluble, while the oxidized Fe<sup>3+</sup> state (ferric iron) occurs in oxic conditions and is characterized by insolubility (Madigan and Martinko 2006). Bacteria in the aerobic environment encounter the latter, insoluble form, quite often and already that presents a challenge in obtaining iron for the cellular processes. However, for pathogenic bacteria, as well as for any other commensal bacterium the situation is even more challenging. Even though tissues and blood of their hosts, such as mammals, are abundant in iron in amounts high enough to support bacterial growth, the hosts employ a variety of iron-binding proteins to limit the availability of free iron, keeping its levels below those required for microbial growth (Andrews and Schmidt 2007; Litwin and Calderwood 1993; Wooldridge and Williams 1993). These proteins include hemoglobin in erythrocytes, transferrin and also lactoferrin which is the major iron-chelating molecule in mucosal surfaces (Gilbreath et al. 2011). In fact, low levels of iron are a signal for some pathogens for induction of virulence genes (Andrews and Schmidt 2007; Litwin and Calderwood 1993).

#### 1.3.5 Iron acquisition

Pathogens can deal with scarce amounts of iron available to them in several ways. Under conditions where iron is soluble (in the form of ferrous ion Fe<sup>2+</sup>) they can employ various iron uptake systems. In Gram-negative bacteria such a system is typically composed of a specific outer membrane receptor, a periplasmic binding protein as well as a transporter protein coupled with an ATP-hydrolyzing protein, both of which are present in the inner membrane (Clarke et al. 2001). In *C. jejuni* several iron uptake systems have been characterized, summarized in Table 1-1.

Under conditions where iron is abundant, but not readily available for the bacteria, specialized molecules called siderophores are employed to acquire the bound iron from host proteins. Siderophores are high-affinity chelating agents of low molecular weight whose role is to

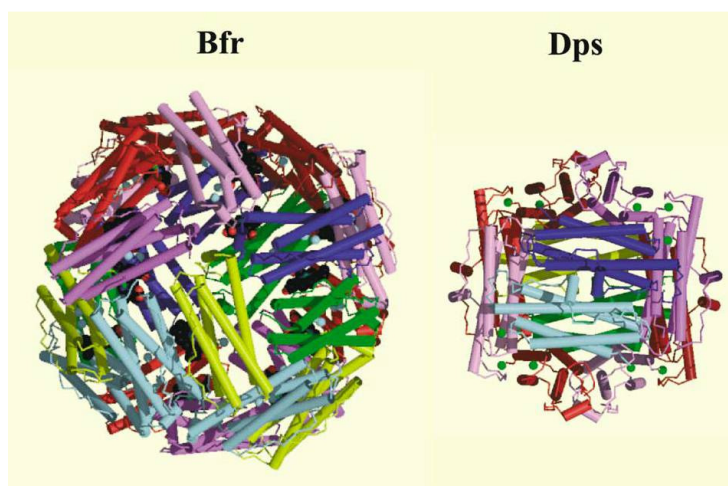
bind the ferric ion from the environment and make it accessible (soluble) to bacterial cell (Neilands 1995). Bacteria produce a wide variety of these molecules (Wooldridge and Williams 1993) and it is common for a bacterium to have siderophore uptake systems that are specific for other types of siderophores than the bacterium can synthesize (Clarke et al. 2001). It appears that *C. jejuni* does not synthesize siderophores itself but it can utilize exogenous siderophores from other bacteria (Baig et al. 1986).

**Table 1-1. Summary of identified iron acquisition systems in *C. jejuni* and *C. coli***

<b>Name</b>	<b>Type</b>	<b>Reference</b>
FeoB	Fe <sup>2+</sup> transport system	(van Vliet et al. 2002) (Naikare et al. 2006)
ChuA, Cj1613c	Hemin/hemoglobin utilization	(Ridley et al. 2006)
CeuBCDE	Enterochelin transport system	(Richardson and Park 1995)
CfrA	Ferric enterobactin uptake receptor	(Guerry et al. 1997)
CfhuABD	Ferrichrome uptake system	(Galindo et al. 2001)
Cj0173-0178	Transferrin / lactoferrin uptake	(Miller et al. 2008)
Cj0178 (CtuA)	system	
p19, ftr1	High-affinity iron uptake system	(Chan et al. 2010)

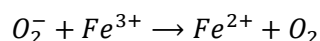
### 1.3.6 Iron storage

Another way for bacteria to deal with iron deficiency is by storing the iron inside specialized proteins to be able to release it when the amount of iron in the environment is scarce. There are three general types of iron-storing proteins known in bacteria: the archetypal ferritins, the haem-containing bacterioferritins and the Dps proteins, all of which show some resemblance in their structural and functional features (Andrews et al. 2003). The shape of these proteins, a spherical shell consisting of either 24 (in ferritins and bacterioferritins) or 12 subunits (Dps proteins) with a hollow core is what allows them to bind, oxidize and store iron efficiently, protecting it from reacting with other proteins or chemical compounds in the cell. Figure 1-11 shows the slight differences in structure of a bacterioferritin (Bfr) and a Dps protein from *E. coli*.

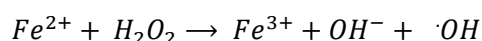


**Figure 1-11. Structures of the bacterioferritin (24-mers) and Dps protein (12-mer) from *E. coli* (Andrews et al. 2003).**

Storing ferrous iron and thereby keeping it isolated has an additional advantage for a bacterial cell. Free iron can namely interact with ROS such as hydrogen peroxide and superoxide which leads to formation of the most reactive and toxic of all ROS – the hydroxyl radical. When in ferric form, iron can interact with superoxide and be reduced to divalent ferrous iron as visualized in Haber-Weiss reaction (Cornelis et al. 2011).



Iron in its ferrous form on the other hand, can be oxidized in a Fenton reaction to form hydroxyl radical:



Therefore, it is critical for a cell to maintain iron homeostasis and for that reason processes such as iron uptake or iron storage need to be regulated.

### 1.3.7 Regulation of iron uptake by Fur

The major gene regulating the processes of iron uptake in bacteria is Fur (ferric uptake regulator) and this regulation occurs at the transcriptional level (Gilbreath et al. 2011). Fur protein is abundant in many Gram positive, as well as Gram negative bacteria and it consists of two domains (Hantke 2001). While the N-terminal part has a role in DNA binding, the C-terminal part is responsible for metal binding and oligomerization. In *E. coli* Fur requires ferrous iron or other divalent metal ions as cofactors to activate Fur as a repressor of iron transport systems (Bagg and Neilands 1987). Fur represses by binding to a specific recognition sequence, termed the Fur-box,

the consensus of which has been widely discussed and altered over time from 19-bp inverted repeat sequence 5'-GATAATGATAATCATTATC-3' (de Lorenzo et al. 1987) to a binding site consisting of three hexameric repeats of GATAAT (Escobar et al. 1998). The most recent reinterpretation of the Fur box sequence is a 15-bp inverted heptamer repeat (7-1-7) which in fact includes both of the previous suggestions (Baichoo and Helmann 2002).

*C. jejuni* Fur protein shares 35% similarity with the one from *E. coli* (Wooldridge et al. 1994). Furthermore, *C. jejuni* Fur was capable of partial functional complementation of its homolog in *E. coli*, suggesting some similarity in the Fur recognition sequences between these two pathogens. An analysis of transcriptome of *C. jejuni* in response to iron availability, as well as transcriptome of a *fur* mutant, allowed for identification of 647 genes whose transcripts were altered in response to iron and 53 Fur-regulated genes (Palyada et al. 2004). Subsequent analysis of the promoter regions of the most iron-repressed and Fur-regulated genes revealed a putative Fur binding consensus sequence, which is presented in Figure 1-12 and in fact differs from the one known from *E. coli*. In order to confirm this sequence, additional experiments including DNA footprinting are necessary.

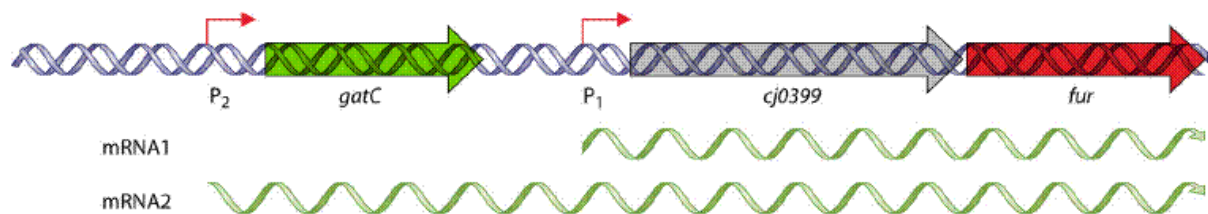


**Figure 1-12. Sequence logo of the putative Fur binding site (Palyada et al. 2004).** The height of each letter corresponds to the relative frequency of that base at that position. The height of the letter corresponds to the sequence conservation at the given position.

It is worth mentioning here that the high number of genes found to be affected by iron availability in this study indicates that iron affects global transcription of genes in *C. jejuni*. The authors also discovered that there was a significant difference between the transcriptional response of *C. jejuni* to a sudden addition of iron and the transcriptome during steady-state growth in the presence of iron at mid-log phase, with a larger number of genes being affected after sudden addition of iron. Therefore, when planning experiments, one should always consider whether one wishes to investigate the adaptation of *C. jejuni* to sudden addition of iron or a general steady-state transcriptome in the presence of iron. The majority of transcript levels that were changed while

investigating the adaptation of *C. jejuni* to iron in the course of 15 minutes, and not during steady-state growth, belonged to functional categories of energy metabolism, ribosomal protein synthesis and modification, surface structures and hypothetical proteins. Transcript levels that were affected during both conditions were classified as belonging to the category of transport and binding proteins.

Curiously, Fur was not identified as one of the genes being affected by iron in neither of the two conditions, which supports the previously reported absence of iron-responsive autoregulation of *C. jejuni* Fur, also common in other bacteria (van Vliet et al. 2000). In many other Gram negative bacteria *fur* has its own promoter located directly upstream of the gene, whereas in *C. jejuni* *fur* expression is controlled by one of two distal promoters, as illustrated in Figure 1-13 (Gilbreath et al. 2011).



**Figure 1-13. Genetic organization of the *C. jejuni fur* locus (Gilbreath et al. 2011).** Expression of the *fur* gene is controlled by one of two distal promoters. If transcription is initiated from the P<sub>1</sub> promoter, a polycistronic mRNA containing both *cj0399* and *fur* coding regions is produced (mRNA<sub>1</sub>). If the transcription is initiated from the P<sub>2</sub> promoter, mRNA carrying *gatC*, *cj0399* and *fur* is produced (mRNA<sub>2</sub>).

Neither of the two promoters responds to the level of iron or Fur and therefore it was suggested that they can be regulated by unknown environmental stimuli.

Among the 53 genes identified as Fur-regulated were ferredoxin *fdxA* involved in oxidative stress, genes involved in flagella biogenesis as well as genes involved in iron acquisition and transport that were repressed in response to both Fur and iron (Palyada et al. 2004). These results were confirmed in a similar study published in parallel that identified genes responsive to iron in *C. jejuni* and the Fur regulon both at transcriptional and translational level (Holmes et al. 2005).

### 1.3.8 Iron-responsive regulation by PerR during oxidative stress

A gene associated with oxidative stress response, *perR* (peroxide stress regulator) was found to be upregulated under iron-limited conditions and downregulated after supplementing medium with iron in two microarray studies (Holmes et al. 2005;Palyada et al. 2004). PerR is a member of a Fur



superfamily of metalloregulatory proteins that are characterized by being small, dimeric and DNA-binding proteins that respond to metal ions (Mongkolsuk and Helmann 2002). PerR is very well characterized in *Bacillus subtilis* (Zuber 2009), where it is a repressor of oxidative stress genes such as *kata*, *ahpCF* and *mrgA* (Dps-like protein) when it binds a single ferrous atom (Imlay 2008), (Zuber 2009). During oxidative stress, PerR senses peroxides such as  $H_2O_2$  by metal catalyzed oxidation (MCO) (Lee and Helmann 2006).  $H_2O_2$  oxidizes the bound ferrous ion in the previously mentioned Fenton reaction, generating hydroxyl radical. This leads to protein oxidation and incorporation of oxygen atom into one of two histidine residues responsible for binding the ferrous iron, which, in turn, leads to the release of ferric iron and prevents binding of a new ferrous iron (Imlay 2008; Lee and Helmann 2006). In a metal-free form, PerR is incapable of binding DNA and hence, the repression of oxidative stress genes regulated by it is relieved.

In *C. jejuni* a Fur homologue identified as PerR has been identified. It is the first Gram negative bacterium described to have a peroxide stress defense regulated by PerR instead of the common oxidative stress regulator OxyR (van Vliet et al. 1999). *C. jejuni* PerR protein was found to share 32% homology with *B. subtilis* PerR and 37% homology with *C. jejuni* Fur (van Vliet et al. 2002). Characterization of a *perR* deficient mutant identified PerR as an iron-dependent repressor of peroxide stress genes *kata* and *ahpC*. The *perR* mutant was shown to be hyper-resistant to peroxide stress and in a double *perR fur* mutant the expression of known iron-repressed genes was derepressed. Therefore it was suggested that *C. jejuni* PerR is a functional, but nonhomologous substitution for the OxyR protein, common in other Gram negative bacteria (Christman et al. 1989).

The binding sequence of PerR in *B. subtilis* was suggested to have a consensus of 5'-CTAtnTTAtAATnATTATAAattA-3' (n - any nucleotide, lower case letters - less conserved nucleotide) (Chen et al. 1995). This sequence was further confirmed by identification of PerR binding sites in front of genes known to be regulated by PerR (Herbig and Helmann 2001). Based on similarity to that sequence, the consensus sequence of *C. jejuni* Fur box as well as PerR box sequence from *S. aureus* (Horsburgh et al. 2001), PerR-box was identified in front of several *C. jejuni* genes, including *ahpC*, *kata*, *dps*, *sodB* as well as *perR* itself, indicating the possibility of autoregulation (van Vliet et al. 2002). Figure 1-14 presents results of analysis of *C. jejuni* 11168 genome in search for genes with the putative Fur/PerR boxes.

(A) *C. jejuni* Fur-box analysis

		Consensus nATnATnATnATnATn			RBS	Start
<b>Iron transport</b>						
Cj0178 <sup>a</sup>	TAAAT	TTTGATAATAATTATAAAT	AATTA	[ 73 bp]	AGAAAGTAACAATAATAATG	
CeuB	TCATT	TTTGATAATTATTATTACT	AT-----		AAGGAAAGATTG	
CfrA	AAATT	AATAATTATTATCATAATA	TTTTG	[ 23 bp]	AAGGAGAAAAATG	
ChuA	TTCTA	AATTATTATGATATAAAAT	ATCAT	[ 9 bp]	AAGGAGTAAAATTTG	
ChuA	ATTAT	GATATAAAATTATCATTATT	TATTT	[ 5 bp]	AAGGAGTAAAATTTG	
p19 <sup>a</sup>	ATTTT	GATAATAAATATTATTTT	ATCAA	[ 5 bp]	AAGGATAATGATAATG	
ExbB	TTAAA	AATTATGATAATTGATATT	AATTA	[ 51 bp]	TGGAGAAAAATATG	
FeoB <sup>b</sup>	TTTTT	TATTATAATATTATTATT	AATT-----		TAGGAATTACAATG	
CfhA	ATCTA	AATGATAAAAAATTACTATT	TAGTT	[ 67 bp]	GGGAGAGAAGATG	
<b>Iron storage/binding</b>						
Cft	TTTTT	CTTAANTATAATTTTAAAC	TATCA	[ 30 bp]	AAGGAGATAAGAATG	
Dps/Bfr <sup>b</sup>	TAATT	AATAATAATAATTATTATT	TAAGA	[ 30 bp]	AAGGAGTCAATCATG	
Cj0241c	ACTTT	AATAATAATAATTTTATA	TAATT	[ 58 bp]	AAAAGTACTCAAAACCATG	

(B) *C. jejuni* PerR-box analysis

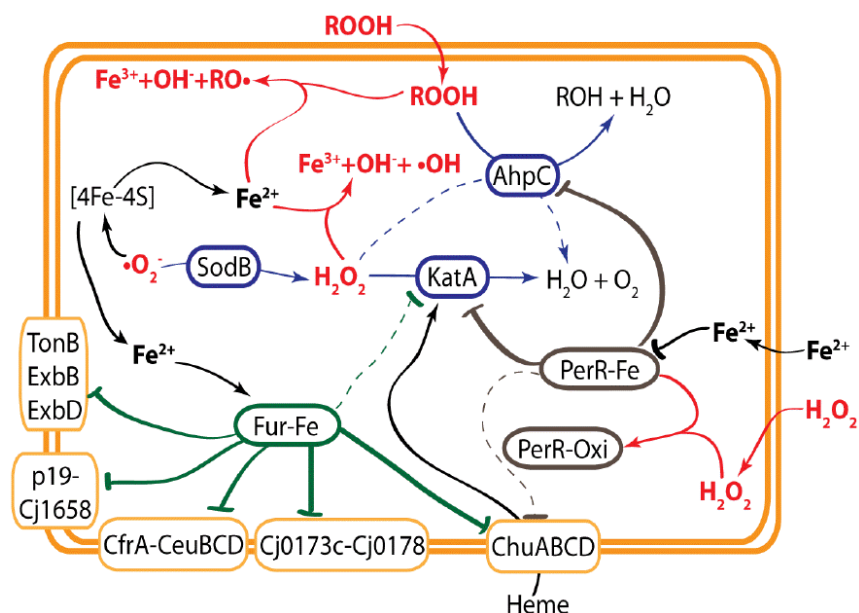
		Consensus nATnATnATnATnATn			RBS	Start
Fur		TTAnAAATnATTAT				
PerR Bs <sup>b</sup>		atTAtAATTTATTATaAt				
PerR Sa <sup>c</sup>						
AhpC	CCTTA	TTTGATAATAATTATAACT	TTTTT	[ 87 bp]	TAGGAGAAAAATATG	
KatA	ATTTT	GATAATTATTATTATAATA	TAAAA	[101 bp]	TAGGAGAAAAACAATG	
TrxB	TACCA	AATAATAATTTTATTAAAC	TACTA	[ 45 bp]	AAGGAAATATAAATG	
HemA <sup>a</sup>	ATTTA	AATTTTAATTATTTTAATT	TAGCT	[ 43 bp]	AAGGAAAATTAATG	
Dps/Bfr <sup>d</sup>	TAATT	AATAATAATAATTATTATT	TAAGA	[ 30 bp]	AAGGAGTCAATCATG	
SodB	TTTAA	TATTATTATGGTAATATT	TCAAT	[ 15 bp]	AAGGAGAAATTATG	
Ccp	TTTTT	AATAATATTATTTTTATT	TAAGC	[ 37 bp]	TAGGAGTTTATAAATG	
PerR	CATTA	AATAATAAAAAATTATTATT	TAATA	[ 48 bp]	AAGGAATAAATAAATG	

**Figure 1-14. Analysis of the *C. jejuni* NCTC11168 genome sequence for putative Fur/PerR boxes (modified from v. Vliet et al. 2002).** A: Fur boxes present in promoters of genes functioning in iron transport or iron storage. B: PerR boxes present in promoters of genes functioning in oxidative stress defense.

As seen in Figure 1-14, there is a significant similarity between the PerR and Fur boxes, which makes it difficult to distinguish between PerR and Fur regulons based on the sequences alone. Experimental binding studies should solve this problem.

The oxidative stress stimulon, as well as PerR regulon in *C. jejuni* was characterized in a large study investigating *C. jejuni* transcriptome supplemented with phenotypic analysis of selected regulatory mutants (Palyada et al. 2009). Genes with altered transcription were identified in the wild type, as well as *perR* and *fur* mutants in response to various conditions, including different types of oxidative stress in iron-rich and iron-limited medium. A total of 104 genes were identified as members of the PerR regulon in that study. Among genes repressed by PerR were, unsurprisingly, oxidative stress response genes *kataA*, *ahpC*, *cft* and *dps*. Among genes found to be repressed by PerR under iron-restricted conditions were genes encoding proteins that take part in iron acquisition systems. Several other genes were also found to be activated by PerR, however, since their transcripts were lower also in response to oxidative stress; it is probable that the response seen was the effect of oxidative damage. Interestingly, *perR* itself also belonged to this group, confirming the possibility for autoregulation suggested earlier. This study revealed an overlap between PerR and Fur regulon, which is likely to be caused by the previously mentioned similarity in recognition sequences of these proteins. A number of genes were identified that were positively regulated by PerR and/or repressed by Fur. Among these genes were those encoding proteins involved in energy metabolism, genes encoding proteins with iron-sulfur clusters or iron and others. One oxidative stress gene, *sodB*, was also classified to belong to this group.

These extensive data, combined with the analysis of oxidative stress stimulon, allowed the authors of that study to identify or confirm links between the genes involved in oxidative stress response and iron metabolism, which they depicted in Figure 1-15.



**Figure 1-15. Schematic representation of oxidative stress metabolism and regulatory network in *C. jejuni* (Palyada et al. 2009).** The oxidants are in red color, the anti-oxidant proteins are in blue boxes and the transcriptional regulators, Fur and PerR, are in green and gray boxes, respectively. SodB catalyzes the dismutation of superoxide anion ( $\cdot\text{O}_2^-$ ) to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). KatA reduces  $\text{H}_2\text{O}_2$  to water and oxygen. AhpC catalyzes the reduction of organic hydroperoxide (ROOH) and  $\text{H}_2\text{O}_2$  to their corresponding alcohols. Dashed lines indicate secondary catalysis or repression. Intracellular iron reacts with  $\text{H}_2\text{O}_2$  and ROOH to produce hydroxyl radicals ( $\cdot\text{OH}$ ) and alkoxyl radicals ( $\text{RO}\cdot$ ), which are the most toxic ROS. Iron-bound Fur (Fur-Fe) is the primary repressor of iron-acquisition pathways (green lines) and the secondary repressor of KatA. Iron-bound PerR is the primary repressor of KatA and AhpC (gray lines) and the secondary repressor of ChuABCD (dashed gray line).  $\text{H}_2\text{O}_2$  makes PerR unable to act as a repressor. Iron represses PerR through an unidentified pathway. Heme is transported by ChuABCD and is required for KatA activity.

The key finding that comes from that study is the importance of iron in the oxidative stress response. Not only is iron the main catalyst required for formation of toxic hydroxyl radicals, but it also plays a major role in regulating expression of genes involved both in oxidative stress as well as in iron metabolism.

### 1.3.9 Dps protein – protein with a dual role

One of the genes that were reported to be PerR repressed in *C. jejuni* was *dps* (Palyada et al. 2009), a gene coding for a Dps protein (DNA-binding protein from starved cells). It was discovered in *E. coli* in 1992 and described as a starvation-induced protein capable of unspecific binding of DNA and important for protection against peroxide stress (Almiron et al. 1992). Later it was revealed that Dps can also bind and store  $\text{Fe}^{2+}$ , thereby preventing hydroxyl radical generation and hence

oxidative damage to the cell (Zhao et al. 2002). It appeared therefore that by combining the DNA-binding property and iron storing, Dps protein can protect a cell in dual way.

Owing to its ferritin-like structure described earlier (Figure 1-11), Dps can bind, oxidize and store iron, which seems to be the main mechanism by which this protein protects cells during oxidative stress. As the name implies, Dps is generally considered a protein induced during starvation or upon entry into stationary phase. However, conditions for induction of the protein vary greatly among the bacterial species and are dependent on the growth phase and the type of stress. In *E. coli* the Dps protein was found to protect the stationary phase cells from oxidative stress as well as various other types of stress (see Table 1-2) (Almiron et al. 1992; Martinez and Kolter 1997; Nair and Finkel 2004). It was also shown that the induction of the protein occurs upon entry into stationary phase, reaching approximately 20000 monomers after 1 day and is governed by RpoS, whereas in exponential phase it is almost undetectable (Almiron et al. 1992). Significant differences in regulation of this protein were reported between growth phases. In exponential phase, Dps induction occurred upon treatment with H<sub>2</sub>O<sub>2</sub> and it was governed by OxyR regulator, whereas in stationary phase the induction was dependent on RpoS transcription factor and the histone-like IHF protein (Altuvia et al. 1994). Dps was also shown to play a major role in condensing DNA during stationary phase (Nair and Finkel 2004). Later it was discovered that two additional nucleoid-associated proteins, FIS and H-NS selectively repress the *dps* gene influencing binding of transcription factors to the *dps* promoter region (Grainger et al. 2008).

### **1.3.10 Role and regulation of the Dps protein in different bacteria**

Since the discovery of the Dps protein in *E. coli* (Almiron et al. 1992), homologues of the gene encoding this protein were identified in many other bacteria. Selected bacterial species in which the Dps protein has been characterized are summarized in Table 1-2 together with the so far identified properties of each of the homologues.

**Table 1-2. Known characteristics of Dps proteins from selected bacteria**

<b>Bacterial species</b>	<b>DNA binding</b>	<b>Type of stress prevented</b>	<b>Reference</b>
<i>Escherichia coli</i> Dps	Yes	Oxidative stress UV and gamma irradiation Iron and copper toxicity Thermal stress Acid and base stress	(Almiron et al. 1992) (Choi et al. 2000) (Nair and Finkel 2004) (Ohniwa et al. 2006) (Jeong et al. 2008)
<i>Salmonella enterica</i> serovar Typhimurium	Not reported	Peroxide stress	(Halsey et al. 2004) (Calhoun and Kwon 2011)
<i>Helicobacter pylori</i> NapA	Yes	Oxidative stress Acid stress	(Cooksley et al. 2003) (Wen et al. 2003) (Wang et al. 2006) (Ceci et al. 2007)
<i>Campylobacter jejuni</i> Dps	No	Peroxide stress	(Ishikawa et al. 2003)
<i>Staphylococcus aureus</i> MrgA	Yes	Oxidative stress UV radiation	(Morikawa et al. 2006) (Morikawa et al. 2007)
<i>Bacillus subtilis</i> MrgA and MrgC	Yes	Peroxide stress Glucose starvation Heat and osmotic stress Metal ions limitation Ethanol stress	(Chen et al. 1995) (Chen et al. 1993) (Antelmann et al. 1997)
<i>Listeria monocytogenes</i> Fri	Not reported	Oxidative stress Heat and cold shock	(Dussurget et al. 2005) (Olsen et al. 2005)
<i>Streptococcus mutans</i> Dpr	No	Oxidative stress	(Yamamoto et al. 2004)
<i>Synechococcus</i> sp. DpsA	Yes	Peroxide stress	(Pena and Bullerjahn 1995)
<i>Mycobacterium smegmatis</i> Dps1 and Dps2	Yes	Peroxide stress Glucose starvation Carbon starvation	(Gupta and Chatterji 2003) (Ghatak et al. 2011) (Gupta et al. 2002)
<i>Lactococcus lactis</i> DpsA and DpsB	Yes	Oxidative stress	(Stillman et al. 2005)
<i>Deinococcus radiodurans</i> Dps-1	Yes	Oxidative stress	(Bhattacharyya and Grove 2007)

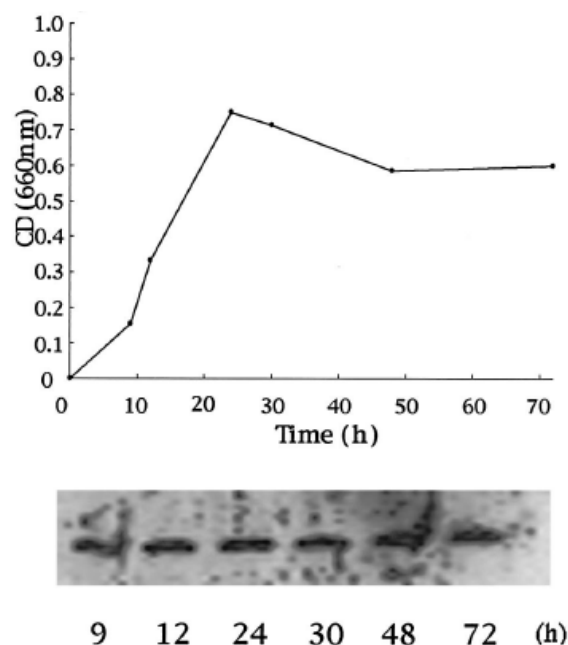
In *L. monocytogenes* the Dps homologue Fri was found to protect cells from oxidative stress both in exponential and stationary growth phases, however, the *fri* mutant was more sensitive to oxidative stress in exponential phase (Olsen et al. 2005). The importance of the protein for survival in late stationary phase was also reported for this bacterium in the same study. The authors'

observations of greater sensitivity to oxidative stress in exponential phase compared to the stationary phase were confirmed in another study reporting that in *L. monocytogenes* *fri* is only transcribed in exponential phase, but because of long half-life of Dps protein, significant amounts are still present in stationary phase (Fiorini et al. 2008). The *fri* gene appears to be regulated from three promoters and controlled by PerR, Fur and  $\sigma^B$  (Fiorini et al. 2008;Olsen et al. 2005).

The induction and regulation of Dps homologue MrgA in the Gram positive pathogen *S. aureus* differs from *E. coli*. First of all, no nucleoid compaction was reported in stationary phase and it was attributed to the lack of IHF that in *E. coli* regulates Dps expression (Morikawa et al. 2006). MrgA induction as well as nucleoid compaction was, however, observed in mid-log phase in response to oxidative stress caused by exposure to specific oxidative agents, including H<sub>2</sub>O<sub>2</sub>. PerR was identified as MrgA repressor in *S. aureus*. In *Salmonella enterica* serovar Typhimurium Dps expression was shown to be governed by OxyR, Fur and RpoS and the mutant cells were more sensitive to oxidative stress in stationary phase compared to exponential phase (Lacour and Landini 2004;Yoo et al. 2007). In *H. pylori*, the closest relative to *C. jejuni*, the Dps homologue termed NapA (neutrophil-activating protein) was shown to increase in level in stationary phase and its expression is controlled by Fur (Cooksley et al. 2003). Aggregation of DNA mediated by NapA was not shown to be growth phase dependent, but dependent on acidic pH, which reflects the unique niche of *H. pylori* (Ceci et al. 2007).

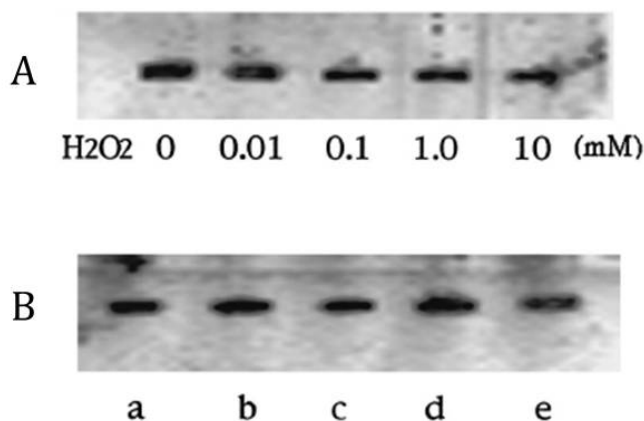
#### **1.3.11 Role and regulation of *C. jejuni* Dps**

*C. jejuni* does not have the stationary phase transcriptional factor RpoS, the OxyR regulator or the nucleoid protein Fis and for this reason Dps regulation cannot be dependent on these proteins (Ohniwa et al. 2006). Dps from *C. jejuni* was found to be required for peroxide stress resistance when both growing cells as well as overnight culture were stressed, however, differences between sensitivity to stress between growth phases were not investigated (Ishikawa et al. 2003). *C. jejuni* Dps is capable of binding up to 40 atoms of iron per monomer and investigation of the effect of the iron chelator Desferal on survival during stress revealed that the iron storing property of Dps is sufficient to prevent cells from dying under oxidative stress. Additionally, Dps protein was shown to be constitutively expressed independently of the growth phase (Figure 1-16).



**Figure 1-16. Growth curve of *C. jejuni* 81-176 and Dps protein production during growth (9 to 72 h) (modified from Ishikawa et al 2003).**

Similarly, no induction of Dps was observed when cells were subjected to various concentrations of H<sub>2</sub>O<sub>2</sub> or varying iron concentrations (Figure 1-17).



**Figure 1-17. Production of *C. jejuni* Dps protein under oxidative stress conditions and in iron-rich and iron-limited conditions (modified from Ishikawa et al 2003).** (A) Dps synthesis after exposure to different H<sub>2</sub>O<sub>2</sub> concentrations for 10 minutes. (B) Dps synthesis under iron-rich and iron-limited conditions after 24h of growth: a, 20  $\mu$ M Desferal; b, 10  $\mu$ M Desferal; c, normal growth conditions; d, 20  $\mu$ M ferrous sulfate; e, 40  $\mu$ M ferrous sulfate.



Studies that investigated transcriptome and proteome of *C. jejuni* during oxidative stress as well as in iron-rich and iron-limited conditions identified Dps as a member of PerR regulon (Palyada et al. 2009) but not Fur regulon (Holmes et al. 2005). Dps contains recognition sequences for both PerR and Fur (van Vliet et al. 2002) and because of large similarity of these sequences, it is likely that both of these regulators can bind to the sequence or even compete with each other. Very recently, two new oxidative stress regulators were identified in *C. jejuni*. One of them was CosR, specific to  $\epsilon$ -proteobacteria and it was revealed as a repressor of Dps together with other proteins such as SodB, Rrc or LuxS and at the same time a positive regulator of AhpC (Hwang et al. 2011). The *dps* gene together with *sodB*, *kataA*, *ahpC* and *perR* were also identified as members of the regulon of a novel oxidative stress regulator in *C. jejuni*, annotated *cj1556* (Gundogdu et al. 2011). While *sodB* gene was upregulated in the absence of the Cj1556 regulator, the other above mentioned genes, including *dps*, were downregulated.

So far the regulation of the *C. jejuni* Dps protein has been investigated on transcriptional level. However, in some other pathogens it has been shown that homologues of this protein can be regulated on the post-transcriptional and post-translational levels as well. In *E. coli*, the Dps protein was abundant and stable during starvation and oxidative stress, however, after supplementing the medium with glucose, rapid ClpXP-mediated proteolysis of Dps was observed (Stephani et al. 2003). At the same time, *clpP* and *clpA* were also involved in maintaining Dps synthesis during starvation, indicating a large degree of post-translational control. It is possible that the Clp proteolytic complex also plays a role in regulation of the amount of Dps in *C. jejuni*, as proteomic analysis of a  $\Delta clpP$  mutant of *C. jejuni* 11168 showed approximately 1.5-fold induction of Dps protein in the absence of ClpP (Cohn et al. 2007). In *Salmonella enterica* serovar Typhimurium another form of post-translational regulation was observed for Dps protein. It was found to be glycosylated with mannose, glucose and an unknown sugar residue (Hanna et al. 2007). The fact that the glycosylated form of Dps was detected at the highest level during the transition from exponential to stationary growth phase underlines that the function of this protein differs depending on growth phases. Up to date, Dps glycosylation has not been reported in other organisms.

In *H. pylori* the NapA protein production was shown to be influenced by a post-transcriptional regulator CsrA (carbon starvation regulator) (Barnard et al. 2004). Even though the *napA* transcript was higher in the *csrA* mutant, there was less NapA protein produced under the same conditions. The *csrA* mutant was also investigated in *C. jejuni* and was found to be more sensitive to oxidative stress than the wild type, causing the authors to speculate that this regulator

might influence oxidative stress genes, but further experiments are required to confirm this (Fields and Thompson 2008). Nevertheless, several pieces of evidence suggest that different post-transcriptional and post-translational modifications of Dps protein exist and this opens a field for new experiments.

## **Chapter 2**

### **Manuscript I**

**Growth phase dependent response of *Campylobacter jejuni* to oxidative stress**

## **Growth phase dependent response of *Campylobacter jejuni* to oxidative stress**

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Manuscript in preparation

## Abstract

In this study we investigated the differences in stress response of a food borne pathogen *Campylobacter jejuni* between growth phases based on the role and expression of the oxidative stress protein Dps (DNA-binding protein from starved cells). We observed a novel phenotype where the exponential phase cells lacking Dps had greatly decreased survival under oxidative stress compared to stationary phase. The lack of Dps in exponential phase under exposure to oxidative stress caused overall damage to the main cell components, as shown by DNA and RNA degradation. Comparison of *dps* transcript level and Dps protein level between growth phases under iron-rich and iron-limited conditions revealed that changes in the transcript levels did not always correspond to changes in the protein level, indicating post transcriptional or post translational control. Furthermore, we observed an iron-dependent induction of *dps* transcript level in response to oxidative stress in the exponential phase and an iron-dependent repression of *dps* transcript level in the stationary phase under the same conditions. By comparing transcripts and protein levels of Dps in strains deficient in regulators PerR and Fur we found that the roles of these repressors were growth phase dependent. Both PerR and Fur acted as repressors in exponential phase, whereas in stationary phase Fur still acted as a repressor, while PerR became an activator in the absence of Fur. We observed that the transcription of some oxidative stress genes, such as *katA* and *ahpC* differed between growth phases. Additionally, one of oxidative stress genes, *sodB*, was downregulated in the absence of Dps in stationary phase, suggesting a relation in regulation of these two genes. Overall, our study points out the differences between growth phases in stress response of *C. jejuni* that are visible in differential and complex regulation of Dps protein.

## 2.1 Introduction

When bacterial cells enter the stationary phase of growth (Kolter et al. 1993), this leads to many morphological and physiological changes in the cell, which occur at the cellular and molecular level (Frenkiel-Krispin et al. 2004;Rees et al. 1995) and result in increased resistance to different types of stress, such as oxidative stress, heat shock (Jenkins et al. 1988), osmotic stress (Jolivet-Gougeon et al. 2000) and acid stress (Foster and Spector 1995), compared to the cells in exponential growth phase. In Gram negative bacteria, these changes are governed by a global regulator of stationary phase genes, the alternative sigma factor  $\sigma^S$  (RpoS), encoded by the gene *rpoS* (Lange and Hengge-Aronis 1991;McCann et al. 1991), which is induced at the onset of stationary growth phase and regulates a large number of genes with various functions that increase a cell's resistance to stress (Loewen et al. 1998;Osiriphun et al. 2009;Schellhorn et al. 1998;Vijayakumar et al. 2004). One example of such gene is a morphogene *bolA* (Lange and Hengge-Aronis 1991) that directs changes in the cell structure that lead to transformation of the rod shape to a sphere shape. Other examples include genes involved in cell processes leading to increased stress resistance and adaptation ability such as *katE*, encoding hydroperoxidase II (von Ossowski et al. 1991) or the *dps* gene encoding DNA-binding protein from starved cells (Dps) (Almiron et al. 1992;Ingmer 2010;Zhao et al. 2002), both proteins playing major roles in resistance against oxidative stress. Many homologous genes, as well as novel members of RpoS regulon were identified in other Gram negative pathogens (Ibanez-Ruiz et al. 2000;Osiriphun et al. 2009;Schuster et al. 2004).

The pathogenic, Gram negative bacterium *Campylobacter jejuni* is a microaerophilic organism that is the leading cause of bacterial diarrheal disease worldwide (Allos 2001). Symptoms caused by *C. jejuni* are watery or bloody diarrhea, abdominal pain and fever and are usually self-limiting. Relapses might, however, occur and rare, but severe complications are also associated with illness caused by this bacterium (Allos 2001). It can be isolated in high numbers from the avian gut and contaminated poultry meat is the most common source of *C. jejuni* infections in humans (Humphrey et al. 2007). *C. jejuni* is an unusual Gram negative bacterium, since it does not encode a  $\sigma^S$  factor (Parkhill et al. 2000) and correspondingly, either increased or unchanged sensitivity to different types of stress, such as heat, acid and oxidative stress in stationary phase compared to exponential phase has been reported (Kelly et al. 2001;Martinez-Rodriguez and Mackey 2005). Still, *C. jejuni* encodes some genes that are normally induced during stationary growth phase in other Gram negative bacteria such as the previously mentioned Dps.

In *E. coli*, the Dps protein prevents accumulation of hydroxyl radicals by binding iron (Zhao et al. 2002) and additionally protects the DNA during starvation and oxidative stress by tightly

binding to it in an unspecific manner (Almiron et al. 1992;Frenkiel-Krispin et al. 2001;Martinez and Kolter 1997;Wolf et al. 1999). The Dps in *C. jejuni* is known to be essential for survival under oxidative stress, but the DNA binding ability *in vitro* was not reported for *C. jejuni* (Ishikawa et al. 2003). However, the Dps dodecamer is known to bind 40 atoms of iron per monomer and is required for growth and survival under high concentrations of hydrogen peroxide in an overnight culture (Ishikawa et al. 2003). The same study suggested that Dps may protect *C. jejuni* against peroxide stress by iron binding that prevents hydroxyl radical formation by the Fenton reaction. The levels of Dps protein were similar during all growth phases and the protein was not additionally induced by hydrogen peroxide, regardless of the growth phase and the presence of iron (Ishikawa et al. 2003). Two independent studies investigating the transcriptome and the proteome of *C. jejuni* during oxidative stress as well as in iron-rich and iron-limited conditions identified Dps as a member of the PerR (peroxide stress regulator) regulon (Palyada et al. 2009;van Vliet et al. 1999) but not of the Fur (ferric uptake regulator) regulon (Holmes et al. 2005;van Vliet et al. 2002). However, since PerR and Fur have a high similarity between their recognition sequences (van Vliet et al. 2002) it is possible that their regulon overlaps and that they might even compete for binding sites of the same genes.

Along with Dps, *C. jejuni* synthesizes an array of specialized proteins that deal with deleterious effects of toxic oxygen intermediates, Reactive Oxygen Species (ROS) (Bannister et al. 1987). These proteins include, among others, superoxide dismutase (SodB) (Purdy and Park 1994), catalase (KatA) (Grant and Park 1995), alkyl hydroperoxidase (AhpC) (Baillon et al. 1999) or thiol peroxidase (Tpx) (Atack et al. 2008).

In this study we investigated the possible differences in response to oxidative stress between the growth phases of *C. jejuni*, using the Dps protein as an example of a gene whose regulation is known to be growth phase dependent in other bacteria. We investigated what effects the lack of the protein causes in exponential and stationary phases in *C. jejuni* by comparing the resistance to peroxide stress of the wild type and the  $\Delta dps$  mutant cells from these two growth phases and we assessed whether the lack of the protein is causing damage on molecular level. Furthermore, by comparing *dps* transcripts and the levels of protein produced in the wild type and the mutants lacking either one or both of the regulatory proteins, PerR and Fur, we investigated the effect of these two repressors on regulation of Dps at RNA and protein levels.

## 2.2 Materials and methods

### 2.2.1 Bacterial strains and growth conditions

The bacterial strains and plasmids are listed in Table 2-1. All *C. jejuni* strains were grown at 37°C in microaerobic conditions ( 88% N<sub>2</sub>, 6% O<sub>2</sub> and 6% CO<sub>2</sub>) in BHI broth (Brain Heart Infusion, company), MEM-α medium (Invitrogen, cat. No. 46061) or cultivated on Base II plates with 5% bovine blood. Chloramphenicol (20 µg/ml) and Kanamycin (10 µg/ml) were added to the media for growth of mutant strains. The samples for Northern and Western blots were collected inside a microaerobic bench (Whitley VA1000 workstation, Don Whitley Scientific) in order to avoid any oxidative stress caused by atmospheric oxygen.

**Table 2-1. Bacterial strains used in this study.**

Strain	Genotype	Reference
<i>C. jejuni</i> 11168	Clinical isolate	National Collection of Type Cultures
<i>C. jejuni</i> TB1015	$\Delta dps::cat$	This study
<i>C. jejuni</i> AS216	$\Delta perR::cat$	Palyada et al. 2009
<i>C. jejuni</i> AS230	$\Delta fur::cat$	Palyada et al. 2009
<i>C. jejuni</i> AS232	$\Delta per::kan, \Delta fur::cat$	Palyada et al. 2009
<i>E. coli</i> TOP10F'	F'[ <i>lacI</i> <sup>q</sup> Tn10 (Tet <sup>R</sup> )] <i>mcrA</i> $\Delta(mmr-hsdRMS-mcrBC)$ $\Phi 80lacZ\Delta M15 \Delta lacX74 recA1 araD139 \Delta(ara-leu)7697 galU galK rpsL endA1 nupG$	Invitrogen

**Table 2-2. Plasmids and vectors used in this study**

Plasmid	Characteristics	Reference
pCR2.1-TOPO	Cloning vector, 3931 bp, LacZα fragment, KanR, AmpR	Invitrogen
pRY109	<i>E. coli</i> XL1Blue plasmid containing 800 bp <i>cat</i> -cassette	(Yao et al. 1993)
pCR2.1-TOPO- $\Delta dps$	pCR2.1-TOPO with 1214 bp SOEing <i>dps</i> PCR fragment	This study
pCR2.1-TOPO- $\Delta dps$ - <i>cat</i>	pCR2.1-TOPO with 1214 bp SOEing <i>dps</i> PCR fragment and 843 bp <i>cat</i> cassette	This study



**Table 2-3. Primers used in this study**

Primer name	Primer sequence
SOE612	5'-GCTGACCCAAATTTAAAGCT-3'
SOE1277	5'-AGGCCTAGTCTTTGGATGATAGG-3'
SOE1625	5'-CTATCATCCAAAGACTAGGCCTCCATAAATGATGAGCATC-3'
dps2168	5'-CAACTCATCATGGATGGACC-3'
M13 forward (-40)	5'-GTTTTCCTCCAGTCACGAC-3'
M13 reverse	5'-CAGGAAACAGCTATGAC-3'
Cat-up	5'-GTCCTGAACTCTTCATGTCG-3'
Cat-down	5'-CGTCGACAAGCTTTGAAGG-3'

### 2.2.2 Mutant construction

For the construction of *dps::cat* insertion-deletion mutant, SOEing PCR was used (Horton et al. 1989). Two overlapping fragments were generated using this technique, *dps*-1 of size 671 bp (using primer pair SOE612 and SOE1277) and *dps*-2 of size 543 bp (primer pair SOE1625 and dps2168), containing sequences upstream and downstream of *dps* gene, respectively. A *Stu*I restriction site was introduced into each fragment during amplification. The two fragments were joined by SOEing PCR using primer pair SOE612 and dps2168, resulting in a SOEing  $\Delta$ *dps* fragment of size 1214 bp, verified by gel electrophoresis. Amplification of the fragment with *Taq* polymerase generated A-overhangs and the SOEing  $\Delta$ *dps* fragment was subsequently cloned into pCR2.1-TOPO vector, resulting in pCR2.1-TOPO- $\Delta$ *dps* and transformed into *E. coli* TOP 10F' cells. Eighteen white colonies were screened by colony PCR for the presence of the vector containing the desired insert with vector primer pair M13 forward (-40) and M13 reverse. Plasmids with insert of correct size (1421 bp) were identified and the orientation of the SOEing *dps* fragment was verified by restriction analysis with enzymes *Hind* III, present only on the vector, and *Stu*I, present only in the *dps* fragment. Next, the *cat* resistance cassette was cut out from plasmid pRY109 from *E. coli* XL1Blue with *Sma*I enzyme, resulting in a blunt-ended fragment of size 843 bp. The pCR2.1-TOPO- $\Delta$ *dps* was cut with enzyme *Stu*I, and the linear vector was ligated with the *cat* cassette, creating vector pCR2.1-TOPO- $\Delta$ *dps*-*cat*. This vector was then transformed by electroporation into *C. jejuni* 11168 strain and transformants containing the *dps* fragment with *cat* cassette inserted into the *C. jejuni* genome by homologous recombination were selected for on BaseII plates with 20y

Chloramphenicol. The orientation of *cat* gene was checked in the four isolated transformants and confirmed to be in the same direction as the *dps* gene. Transformant TB1015, containing 348-bp deletion of *dps* and a 800 bp *cat* cassette was chosen for further work.

### **2.2.3 Survival during oxidative stress in nutrient-rich medium**

Fresh cultures of *C. jejuni* 11168 and *C. jejuni* 11168  $\Delta$ *dps* were grown overnight on Base II plates. They were then inoculated in 10 ml BHI broth to OD<sub>600</sub> of 0.05. Two cultures (the wild type and  $\Delta$ *dps::cat* mutant) were allowed to grow to exponential phase (4h) and the other two to stationary phase (24h). One ml of each of the four cultures was transferred to an Eppendorf tube and kept in a heating block at 37°C. 100  $\mu$ l were taken out of each tube, serial dilutions were made and 3 x 10  $\mu$ l of each dilution were spotted on Base II plates to estimate CFU/ml. Next, H<sub>2</sub>O<sub>2</sub> solution was added to each tube to a desired concentration (ranging from 2.5 mM to 1 mM) and from that point samples were taken every 20 minutes for 1 hour. When required, all cultures were pretreated with 80 mM desferrioxamine mesylate (Desferal) for 5 minutes before exposure to H<sub>2</sub>O<sub>2</sub>. At each time point, 100  $\mu$ l of cultures were withdrawn, diluted and spotted. The plates were then incubated for 2 days in microaerobic conditions and CFU/ml was determined for each time point.

### **2.2.4 Sample collection for total RNA isolation**

*C. jejuni* 11168 and *C. jejuni* 11168  $\Delta$ *dps::cat* were grown in 30 ml BHI broth to exponential phase (4h) and in 10 ml BHI broth to stationary (24 h) phase. 2 ml of stationary phase cultures of both strains and 8 ml of exponential phase cultures were transferred into in total twelve 15 ml yellow cap centrifuge tubes, opened and closed inside the bench. Each tube represented one of three conditions, at which the samples for RNA from each strain were taken ("no stress", "5 min. H<sub>2</sub>O<sub>2</sub>" and "10 min. H<sub>2</sub>O<sub>2</sub>"). The exponential phase cells were harvested by centrifuging 8 ml culture for 5 minutes at 10000 rpm and then resuspended in 2 ml BHI broth inside the bench. 100  $\mu$ l was taken from four tubes marked as "no stress" for CFU/ml determination. After that, 4 ml of RNA Protect reagent (Qiagen) was added to the cultures in these tubes in order to stabilize RNA. These samples were then vortexed outside of the bench for 5 seconds, incubated at room temperature for 5 minutes and then immediately centrifuged for 2 minutes at 8228  $\times$  g at 0°C. After discarding the supernatant, the pellets were frozen at -20°C until RNA purification. Inside the microaerobic bench, H<sub>2</sub>O<sub>2</sub> was added to concentration of 2.5 mM to the remaining 8 tubes and samples for CFU/ml determination were taken at time points 5' and 10'. RNA was stabilized using RNA Protect as described above.

### 2.2.5 Sample collection for total RNA isolation under iron-limiting conditions

Fresh cultures of *C. jejuni* 11168, *C. jejuni* 11168  $\Delta perR::cat$ , *C. jejuni* 11168  $\Delta fur::cat$  and *C. jejuni* 11168  $\Delta perR::kan \Delta fur::cam$  were grown overnight on Base II plates. Each strain was inoculated in 125 ml of MEM- $\alpha$  medium to an OD<sub>600</sub> of 0.05. The strains were grown with shaking (240 rpm) in 250 ml baffled flasks to exponential (4h) and stationary (24h) phases inside a microaerobic bench. When cultures were ready, 30 ml of each strain was transferred into four 50 ml centrifuge tubes inside the bench (in total 16 tubes). Each of the tube represented one of the four conditions investigated: “no treatment”, “H<sub>2</sub>O<sub>2</sub>, 10 min.”, “no treatment + Fe<sup>2+</sup>”, “Fe<sup>2+</sup> + H<sub>2</sub>O<sub>2</sub>, 10 min.”. All 16 tubes were centrifuged at 4000 rpm for 15 minutes to concentrate the cells. Each culture was then resuspended in 2 ml MEM- $\alpha$  medium. Then, 40  $\mu$ M FeSO<sub>4</sub> was added to appropriate samples and all the tubes were incubated in the bench for 1 hour. Next, H<sub>2</sub>O<sub>2</sub> was added to the appropriate samples in concentrations from 1 mM to 2.5 mM and all the tubes were incubated for 10 more minutes. In the meantime, 100  $\mu$ l was taken out of each of 16 tubes for CFU/ml determination. After 10 minutes, 4 ml RNA protect reagent was added to each sample, which was then vortexed for 5 seconds and incubated for 5 minutes outside of the bench. All 16 tubes were then centrifuged at 4000 rpm for 10 minutes at 0°C and the pellets were frozen at -20°C.

### 2.2.6 Total RNA isolation

The total RNA was extracted using the RNeasy Mini Kit (Qiagen), following the manufacturer’s instructions, but with slight modifications (Moen et al. 2005). The concentrations and purity of RNA samples were measured using Nanodrop 1000 spectrophotometer and the lack of RNA degradation was confirmed by visualizing the RNA samples on a 1% agarose gel.

### 2.2.7 Northern blot analysis

Between 3 and 5  $\mu$ g of RNA samples were denatured and separated on 1% agarose gel containing EtBr (10 mg/ml). RNA was visualized under UV light and the fragment containing RNA was cut from the gel. RNA was transferred to a HybondN+ nylon membrane by capillary blotting overnight. PCR products of the genes of interest were prepared in advance and radioactively labeled using the Ready-to-Go DNA labeling Beads (Amersham Biosciences) and radioactive phosphorus <sup>32</sup>P isotope. The primers used to amplify probes are shown in Table 2-4. The hybridization was performed at 60°C in 0.5 M Sodium Phosphate buffer at pH 7.2 containing 7% SDS overnight. After washing the radioactive signal was detected with Cyclon Plus Phosphor Imager (PerkinElmer).

**Table 2-4. Primers used to create DNA probes for Northern blot analysis**

Probe name	Primers used	Length of the probe (bp)
dps	dps_for: 5'-CACGAGTACACAGAAAAAG-3' dps_rev: 5'-TAGCGCCTATCATCCAAA-3'	316
sodB	sodB_for: 5'-GGTGATTTTTTGAGTGCTG-3' sodB_rev: 5'-CTAACTGATCCCATGCCT-3'	587
tpx	tpx_for: 5'-AGTAGAAGTTGGAGCAGA-3' tpx_rev: 5'-CCACCAAAAAATGCATCAAG-3'	444
ahpC	ahpC_for: 5'-GGAGCGGTAGTATTCTTTT-3' ahpC_rev: 5'-GCCTTTAGGGTTAGCTTT-3'	456
katA	katA_for: 5'-TGCTGCTTGGGATTTTTG-3' katA_rev: 5'-CTTTTGTCTTCTTTTGGGCT-3'	711

### 2.2.8 DNA fragmentation analysis

The wild type and  $\Delta$ dps::cat mutant strains were grown to exponential (4 h) and stationary (24 h) phases and 100  $\mu$ l was taken out from each culture, diluted and plated to determine CFU/ml. Two times one ml were taken from all four cultures and harvested for 1 min. at maximum speed. One ml of each culture was treated with 2.5 mM H<sub>2</sub>O<sub>2</sub> for one hour and then all cultures were prepared as described before (Wang et al. 2006). Low melting point agarose from SeaPlaque was used.

### 2.2.9 Overexpression and purification of Dps protein from *E. coli*

Dps protein was purified from a pTIS-21 vector kindly donated by Dr. Yoshimitsu Mizunoe (Ishikawa et al. 2003). Plasmid pTIS-21 was transformed into *E. coli* BL21 (DE3) cells (Invitrogen) using the protocol from the manual. Transformants were selected on plates containing 50 $\mu$ g Ampicillin. One of the transformants, called ML301, was chosen for purification of Dps protein. ML301 was grown in 1 L lysogeny broth (LB) containing 50 $\mu$ g Ampicillin until OD<sub>600</sub> of 0.4 was reached. Then the Dps expression was induced by adding 1 mM Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) and cells were incubated further for 2 hours. The culture was then collected by spinning at maximum speed for 10 minutes. Pellets were frozen at -20°C. Next day all pellets were thawed on ice, resuspended in 5 ml lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8.0) and pooled together. One mg/ml lysozyme was added and the cells were lysed for 30 minutes on ice, followed by sonication for 1 minute with 10 second intervals. The

lysate was then centrifuged at maximum speed for 45 minutes and supernatant used for subsequent purification. Ni-NTA Superflow Cartridges were used for manual purification of Dps protein from supernatants according to the manual. Three washing steps were performed with 20, 100 and 150 mM imidazole in washing buffer containing 50 mM  $\text{NaH}_2\text{PO}_4$  and 300 mM NaCl at pH 8.0. The protein was eluted with the same buffer containing 250 mM imidazole.

#### **2.2.10 Anti-Dps antibody production**

Purified Dps protein in the elution buffer was separated on 10% Tris-Bis SDS-PAGE gel (Invitrogen) and the gel was stained with SafeBlue stain (Invitrogen) to visualize the bands. Five large bands of Dps monomer were cut out of the gel and used to immunize New Zealand white rabbits three times (Covalab). Polyclonal serum was obtained after 53 days.

#### **2.2.11 Collection of total protein lysates**

*C. jejuni* 11168 and *C. jejuni* 11168  $\Delta\text{dps}$  were grown to exponential (4h) and stationary (24h) growth phases from a start  $\text{OD}_{600}=0.05$  in 12 ml BHI.  $\text{OD}_{600}$  was measured and the optical density of all cultures was adjusted to the lowest measured  $\text{OD}_{600}$  value to obtain the same number of cells. The cultures were then centrifuged at 10000 rpm for 5 minutes and pellets resuspended in 2 ml BHI. This volume of each culture was then divided into two Eppendorf tubes (1 ml culture in each, in total 8 tubes) and to four of them,  $\text{H}_2\text{O}_2$  at concentrations 2.5 mM were added and all tubes were incubated for 10 minutes at 37°C. The cultures were then centrifuged at 10000 rpm for 5 minutes, washed once in physiological salt solution and resuspended in 500  $\mu\text{l}$  TM buffer (10 mM Tris-HCl, pH 7.5 and 5 mM  $\text{MgCl}_2$ ).  $\text{OD}_{600}$  of each culture was again measured and later used to adjust the amount of cells loaded on the gel. Samples were stored at -20°C until further use.

#### **2.2.12 Collection of total protein lysates under iron limiting conditions**

Fresh cultures of *C. jejuni* 11168, *C. jejuni* 11168  $\Delta\text{perR}::\text{cat}$ , *C. jejuni* 11168  $\Delta\text{fur}::\text{cat}$  and *C. jejuni* 11168  $\Delta\text{perR}::\text{kan}$   $\Delta\text{fur}::\text{cam}$  were grown overnight on Base II plates. Each strain was inoculated in 85 ml of MEM- $\alpha$  medium to an  $\text{OD}_{600}$  of 0.05. The strains were grown with shaking (240 rpm) in 250 ml baffled flasks to exponential (4h) and stationary (24h) phases inside a microaerobic bench.  $\text{OD}_{600}$  was measured of each culture, adjusted to the lowest value, and 20 ml of each culture was transferred into four 50-ml centrifuge tubes (in total 16 tubes). Each of the tube represented one of the four conditions investigated: “no treatment”, “ $\text{H}_2\text{O}_2$ , 10 min.”, “no treatment +  $\text{Fe}^{2+}$ ”, “ $\text{Fe}^{2+}$  +  $\text{H}_2\text{O}_2$ , 10 min.”. All 16 tubes were centrifuged at 4000 rpm for 15 minutes to concentrate the cells. Each

culture was then resuspended in 2 ml MEM- $\alpha$  medium. Then, 40  $\mu$ M FeSO<sub>4</sub> was added to appropriate samples and all the tubes were incubated in the bench for 1 hour. Next, H<sub>2</sub>O<sub>2</sub> in concentrations from 1 mM to 2.5 mM was added to the appropriate samples and all the tubes were incubated for 10 more minutes. In the meantime, 100  $\mu$ l was taken out of each of 16 tubes for CFU/ml determination. After 10 minutes, cultures were centrifuged at 4000 rpm for 15 minutes, pellets were resuspended in 1 ml 0.9% salt solution and transferred to Eppendorf tubes. Samples were centrifuged in a table-top centrifuge at 10000 rpm for 10 minutes and each pellet was resuspended in 500  $\mu$ l TM buffer (10 mM Tris-HCl, pH 7.5 and 5 mM MgCl<sub>2</sub>). The OD<sub>600</sub> of each sample was measured and noted.

### 2.2.13 Western blotting

The amount of samples was adjusted according to cell density measured in TM buffer, so that the same amount was loaded in each well. The samples were boiled at 99°C for 10 minutes under denaturing conditions and immediately cooled on ice. Samples were separated on 10% Tris-Bis gel (Invitrogen) in MES buffer (Invitrogen). The gels were then electroblotted onto PVDF membranes and proteins were detected using anti-Dps primary antibodies at a dilution of 1:1000 using Western Breeze kit with secondary anti-rabbit antibodies (Invitrogen). The bands were visualized using Geliance 1000 Imaging System (Perkin Elmer) and quantified based on the intensity using PerkinElmer software (GeneTools from Syngene).

## 2.3 Results

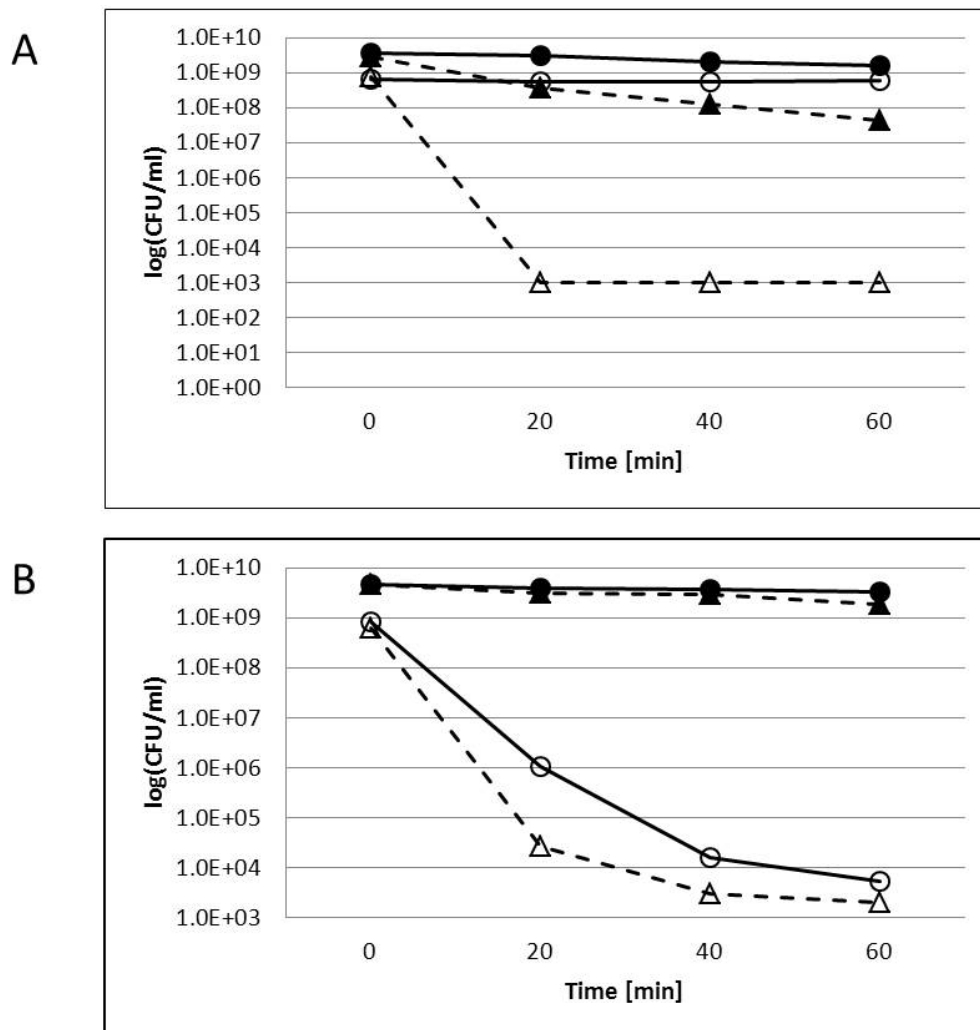
### 2.3.1 Dps protein

Dps protein in many other bacteria such as *E. coli* (Almiron et al. 1992), *B. subtilis* (Chen et al. 1993) or *H. pylori* (Cooksley et al. 2003) is known to be induced in the stationary phase, hence the Dps-mediated protection from oxidative stress in these bacteria is observed mostly in this growth phase. Dps protein is a 149 amino acid long protein encoded by the open reading frame *cj1534c* in *Campylobacter jejuni* NCTC11168 strain. It contains a ferritin-like Pfam domain PF00210 and shows similarity to other members of the Dps protein family, such as NapA from *Helicobacter pylori* (41.3%), MrgA from *Bacillus subtilis* (33.1%) and Fri from *Listeria innocua* (31.8%). Furthermore, Dps protein from *C. jejuni* NCTC11168 is 100% identical to the Dps protein from *C. jejuni* 81-176 (Ishikawa et al. 2003). The analysis of DNA sequence of regions upstream and downstream of the *dps* gene allowed for identification of two putative promoter sequences upstream of the start codon (first pair: 5'-TATAATT-3' and 5'-TTTAAGA-3', second pair: 5'-TATAATA-3' and 5'-TTTAAGTT-3'). A

probable terminator sequence (5'-GGGGC-N<sub>5</sub>-GCCCC-3') was located six nucleotides from the stop codon. This suggests that *dps* is a monocistronic gene and therefore insertion of a resistance cassette in the gene should not affect transcription of any downstream genes. We proceeded with constructing a deletion-insertion mutant in the *dps* gene, where 348 bp of 450 bp were deleted and replaced with a 800 bp *cat* cassette, conferring Chloramphenicol resistance.

### **2.3.2 Dps protein is vital for survival of *C. jejuni* during peroxide stress in exponential phase**

The ability of Dps to prevent formation of ROS in the cells during oxidative stress in different growth phases has not been compared in *C. jejuni* and we decided to investigate whether there are differences in survival during oxidative stress between exponential and stationary growth phases. The wild type strain, *C. jejuni* 11168 (wt) and the mutant strain ( $\Delta$ *dps*) were grown to exponential (4 h) and stationary phases (24 h) and then exposed to hydrogen peroxide (2.5 mM) and cumene hydroperoxide (0.1 mM).



**Figure 2-1. Survival of *C. jejuni* wt and  $\Delta dps$  during exposure to 2.5 mM H<sub>2</sub>O<sub>2</sub> (A) and 0.1 mM CuOOH (B) for 1 hour.** *C. jejuni* 11168 in exponential phase (4h) (○), *C. jejuni*  $\Delta dps$  in exponential phase (Δ), *C. jejuni* 11168 in stationary phase (24h) (●), *C. jejuni*  $\Delta dps$  in stationary phase (▲). Consistent results were obtained from at least three biological replicates.

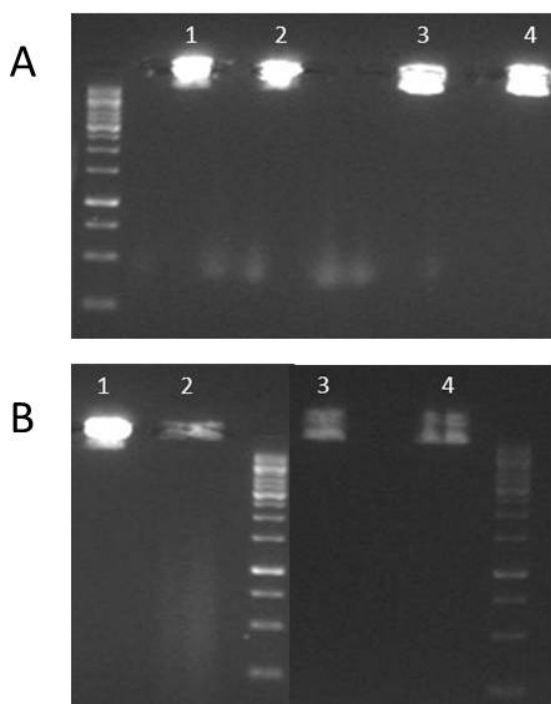
In the absence of the *dps* gene the bacteria were much more susceptible to oxidative stress caused by exposure to hydrogen peroxide in the exponential phase, which was reflected in rapid decrease in cell numbers of the mutant strain compared to stationary phase (Figure 2-1A). A difference in survival between growth phases was also observed when another oxidative agent, cumene hydroperoxide, was used (Figure 2-1B), however, in this case the sensitivity in exponential phase was not Dps-dependent. The *C. jejuni* Dps was shown to prevent the accumulation of toxic hydroxyl radicals in the overnight culture by harvesting iron from the medium (Ishikawa et al. 2003). We have examined whether the iron binding ability of Dps can prevent decrease in survival in both exponential and stationary phases during oxidative stress by adding Desferal to both



cultures prior to hydrogen peroxide exposure. Cultures pretreated with Desferal showed no sensitivity to hydrogen peroxide irrespective of the growth phase, whereas cultures without this treatment rapidly decreased in cell numbers (data not shown).

### 2.3.3 Lack of Dps in exponential phase causes damage of cellular components

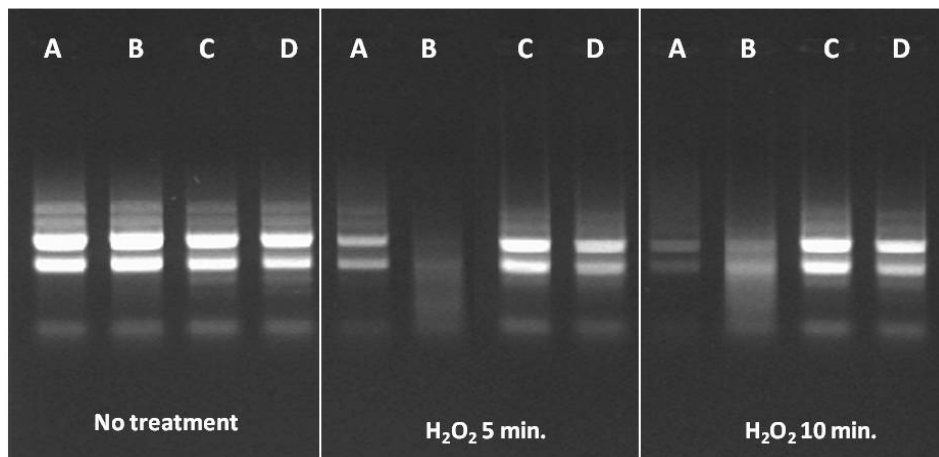
We investigated the extent of damage caused by oxidative stress in *C. jejuni* wild type and  $\Delta dps$  mutant in different growth phases and determined the effect that lack of Dps has on different cellular components such as DNA and RNA. We first visualized the DNA from stress and non-stress conditions in the wild type and  $\Delta dps$  mutant strain and observed DNA degradation only in the mutant in exponential phase under stress conditions (Figure 2-2).



**Figure 2-2. DNA fragmentation analysis of *C. jejuni* 11168 and *C. jejuni*  $\Delta dps$  after 1 hour exposure to 2.5 mM  $H_2O_2$ .** (A) DNA from cells before stress, (B) DNA from cells after stress. Wild type exponential (exp.) phase (1),  $\Delta dps$  exp. phase (2), wild type stationary (st.) phase (3),  $\Delta dps$  st. phase (4).

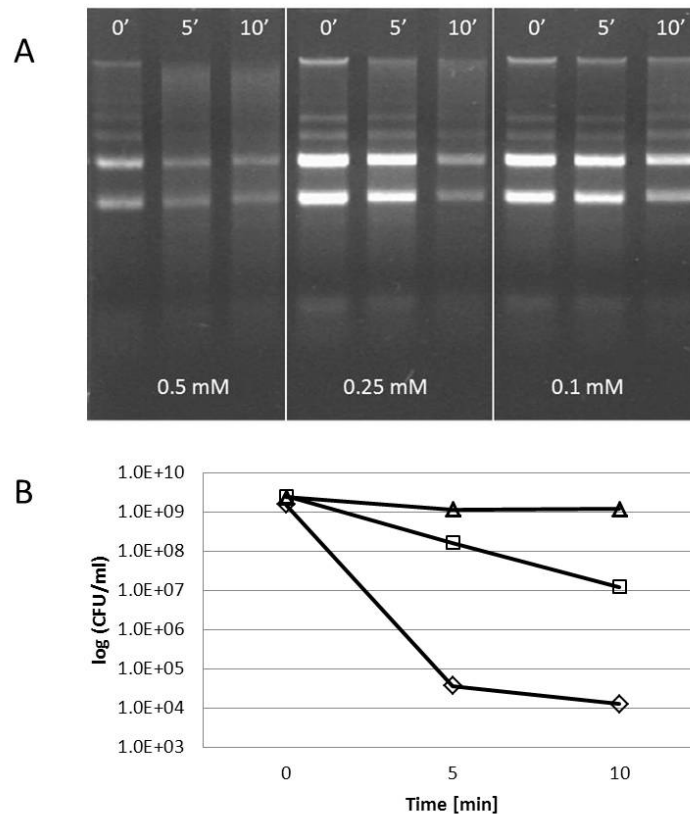
It is worth noting that after 1 hour of exposure to hydrogen peroxide the majority of cells were dead in the *dps* mutant in exponential phase (see Figure 2-1A). Hence, the DNA fragmentation seen in Figure 2-1B, lane 2, is most likely caused by accumulation of hydroxyl radicals caused by the lack of Dps that subsequently has led to cell death.

Next, we investigated whether RNA is damaged when Dps is absent in the cells during oxidative stress. We treated the wild type and the mutant strain in exponential and stationary phases with 2.5 mM H<sub>2</sub>O<sub>2</sub> and purified RNA from cell samples taken before exposure and after 5 and 10 minutes. RNA was degraded only in the mutant from exponential phase exposed to hydrogen peroxide already after the first 5 minutes at this concentration (Figure 2-3).



**Figure 2-3. Degradation of RNA from *C. jejuni* 11168 and *C. jejuni*  $\Delta$ dps in different growth phases after oxidative stress.** Wild type exponential (exp.) phase (A),  $\Delta$ dps exp. phase (B), wild type stationary (st.) phase (C),  $\Delta$ dps st. phase (D).

We also found that RNA damage in the mutant in exponential phase was specifically dependent on hydrogen peroxide concentration. When cells were exposed to lower H<sub>2</sub>O<sub>2</sub> concentrations, no visible RNA damage occurred within 10 minutes at 0.1 mM, whereas already at 0.25 mM, RNA was damaged at the same time point (Figure 2-4). RNA damage correlated with decreasing survival of the cells.

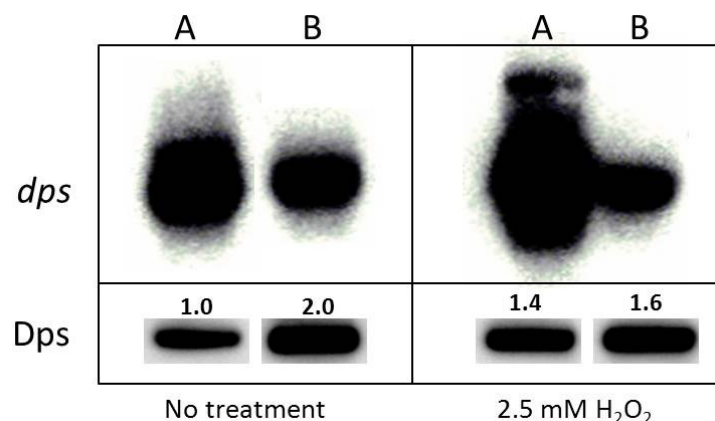


**Figure 2-4. Visualization of RNA damage and corresponding cell counts in *C. jejuni*  $\Delta dps$  from exponential phase (4h) exposed to varying  $H_2O_2$  concentrations. (A) total RNA purified from *C. jejuni*  $\Delta dps$  (B) survival of *C. jejuni*  $\Delta dps$  exposed to different  $H_2O_2$  concentrations: 0.5 mM (diamonds), 0.25 mM (squares), 0.1 mM (triangles).**

These results indicate that without Dps the concentration of the hydroxyl radicals in the cells becomes high enough to cause RNA degradation.

#### 2.3.4 Dps is differentially transcribed and translated in exponential and stationary phases

The observation of differences in sensitivity of the *dps* mutant to hydrogen peroxide stress between growth phases (Figure 2-1) has led us to investigate whether the *dps* transcript level differs between the two growth phases and additionally whether oxidative stress caused by hydrogen peroxide has an effect on the transcription level of *dps*.



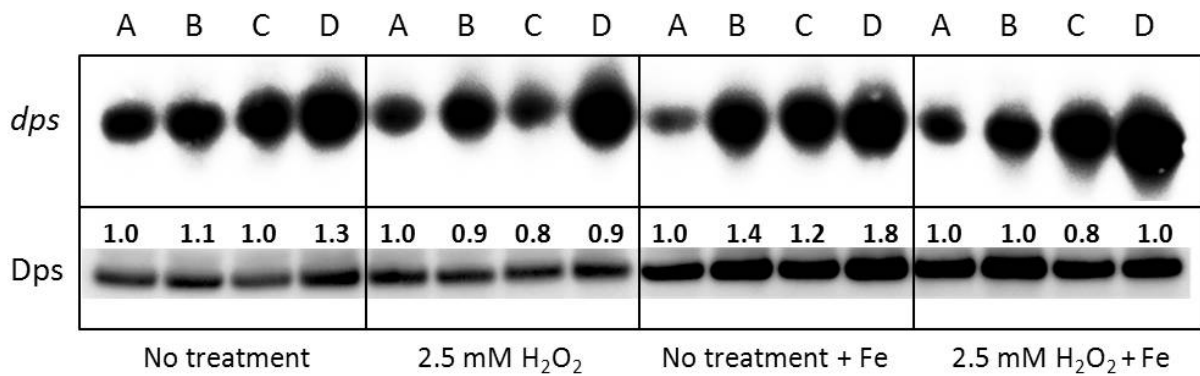
**Figure 2-5. Transcript and translated protein from *dps* in *C. jejuni* 11168 from exponential (4h) and stationary (24) phase cells before and after exposure to 2.5 mM H<sub>2</sub>O<sub>2</sub> for 10 minutes in iron rich conditions.** Wild type exponential phase (A), wild type stationary phase (B). The numbers above the Western blot bands indicate fold changes normalized to the first band (value 1.0), calculated by comparing band intensity using GeneTools software (Syngene). Bands from a representative experiment are shown but the numbers are mean values from two biological replicates.

We observed that the amount of *dps* transcript is slightly higher in the exponential phase than in the stationary phase and it is additionally induced by hydrogen peroxide already after 10 minutes exposure (Figure 2-5). In addition to that, small repression of *dps* transcript is seen in stationary phase after treatment with hydrogen peroxide. To determine whether the observed changes in transcription were also visible at the translation level, we extracted total proteins under the same conditions and performed Western blot using anti-Cj-Dps polyclonal antibodies. Western blot analysis revealed that Dps protein is actually induced twofold in stationary phase compared to exponential phase under no stress conditions (Figure 2-5). Under oxidative stress the level of the Dps protein increases 1.4-fold in exponential phase, whereas in stationary phase the level of the protein is reduced by oxidative stress.

### 2.3.5 PerR and Fur are involved in Dps regulation

Dps was previously shown to be a part of PerR (peroxide stress regulator) regulon in *C. jejuni* by different microarray studies (Holmes et al. 2005; Palyada et al. 2009) and the cultures used in those studies were from mid-log or late exponential growth phase. We investigated *dps* transcription in the early exponential phase cells and the stationary phase cells to determine if control of transcription differs between growth phases. In our analysis we included also Fur (ferric uptake regulator). Dps has not been shown to be a part of Fur regulon, but because of a large similarity of

its recognition sequence to that of PerR's present upstream of *dps* (van Vliet et al. 2002), we considered it to be a possible candidate for control of *dps* transcription. We compared *dps* transcript levels of the wild type with transcript levels of  $\Delta perR$ ,  $\Delta fur$  and  $\Delta perR\Delta fur$  mutants by Northern blot analysis, as well as the amount of Dps protein in the same strains by Western blot analysis. Knowing that iron has an effect on both regulators, activating both PerR and Fur as repressors (Palyada et al. 2009), we used minimal medium with and without iron, thereby affecting activity of either one or both of the regulators.

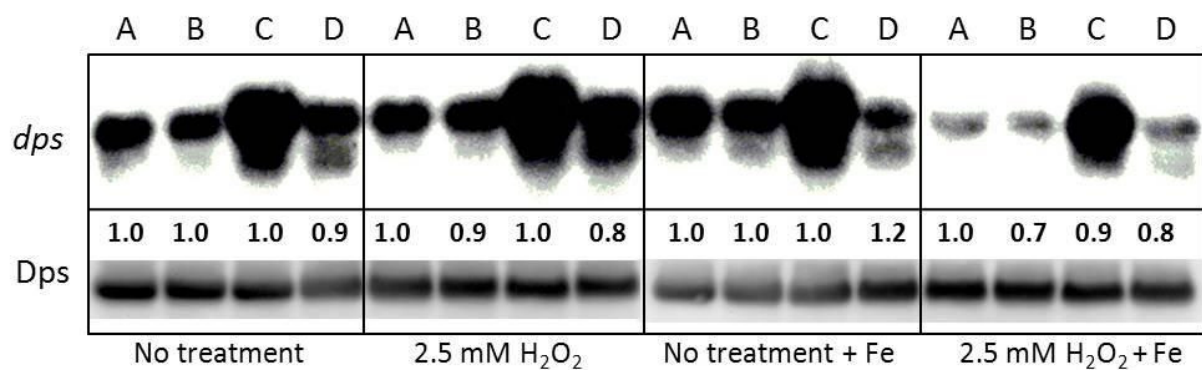


**Figure 2-6. Transcription and translation of *dps* from *C. jejuni* 11168 and mutants lacking regulatory genes from exponential phase.** *C. jejuni* 11168 (A), *C. jejuni*  $\Delta perR$  (B), *C. jejuni*  $\Delta fur$  (C), *C. jejuni*  $\Delta perR\Delta fur$  (D). Incubation with 40  $\mu$ M FeSO<sub>4</sub> lasted 1 hour and incubation with 2.5 mM H<sub>2</sub>O<sub>2</sub> lasted 10 minutes. Northern blots were performed in three biological replicates and a representative result is shown. The numbers above the Western blot bands indicate fold changes normalized to the band of the wild type Dps (value 1.0) separately for each of the four conditions, calculated by comparing band intensity using GeneTools software (Syngene). Bands from a representative experiment are shown but the numbers are mean values from two biological replicates.

Under iron-replete conditions in exponential phase the lack of PerR, Fur or both regulators results in an increase in *dps* transcript level when no treatment is applied, suggesting derepression of *dps* in the single mutants as well as the double mutant (Figure 2-6). A similar increase in protein level is observed in the same samples apart from the  $\Delta fur$  mutant, where no protein induction occurs. When the cultures are treated with hydrogen peroxide, *dps* derepression is still observed in the  $\Delta perR$  mutant and the double mutant, but in the  $\Delta fur$  mutant the *dps* transcript level is repressed. On the protein level a slight repression of the Dps protein is seen in all mutant samples under oxidative stress.

When samples from exponential phase are preincubated with iron, decrease in the *dps* transcript level is observed in the wild type compared to the same sample without iron. The *dps* transcript levels are induced in the single mutants and the double mutant compared to the wild

type level and this induction is also observed on the protein level. Interestingly, when cultures preincubated with iron are stressed with hydrogen peroxide, induction in the *dps* transcript level in the wild type is observed, which corresponds to the transcript level increase presented in our previous result (Figure 2-5), where the transcript levels were examined also under iron-rich conditions. This could indicate dependence of Dps induction during oxidative stress on the presence of iron, but only on transcriptional level, since the Dps protein levels under these conditions remain unchanged, apart from the  $\Delta fur$  mutant, where slight decrease in the protein level is observed.



**Figure 2-7. Transcription and translation of *dps* from *C. jejuni* 11168 and mutants lacking regulatory genes from stationary phase.** *C. jejuni* 11168 (A), *C. jejuni*  $\Delta perR$  (B), *C. jejuni*  $\Delta fur$  (C), *C. jejuni*  $\Delta perRfur$  (D). Incubation with 40  $\mu$ M FeSO<sub>4</sub> lasted 1 hour and incubation with 2.5 mM H<sub>2</sub>O<sub>2</sub> lasted 10 minutes. Northern blots were performed in two biological replicates and a representative result is shown. The numbers above the Western blot bands indicate fold changes normalized to the band of the wild type Dps (value 1.0) separately for each of the four conditions, calculated by comparing band intensity using GeneTools software (Syngene). Bands from a representative experiment are shown but the numbers are mean values from two biological replicates.

Interestingly, in stationary phase cultures under all conditions tested, we observed a high upregulation of *dps* transcript level in the  $\Delta fur$  mutant compared to the level in the wild type and the  $\Delta perR$  mutant (Figure 2-7). No increase in Dps protein level, corresponding to the *dps* transcript increase in the  $\Delta fur$  mutant was, however, observed, suggesting that in stationary phase, *dps* expression could be controlled at a post-transcriptional or post-translational level. While the *dps* transcript level was greatly increased in the  $\Delta fur$  mutant under iron-replete conditions, it was at a similar level in the double mutant as in the wild type. This suggests that Fur acted as a repressor of *dps* in stationary phase, however, when PerR was additionally absent in the double mutant, no de-repression was observed, suggesting that PerR could act as an activator of Dps in the absence of Fur. After treatment with hydrogen peroxide, certain induction in *dps* transcript in the double

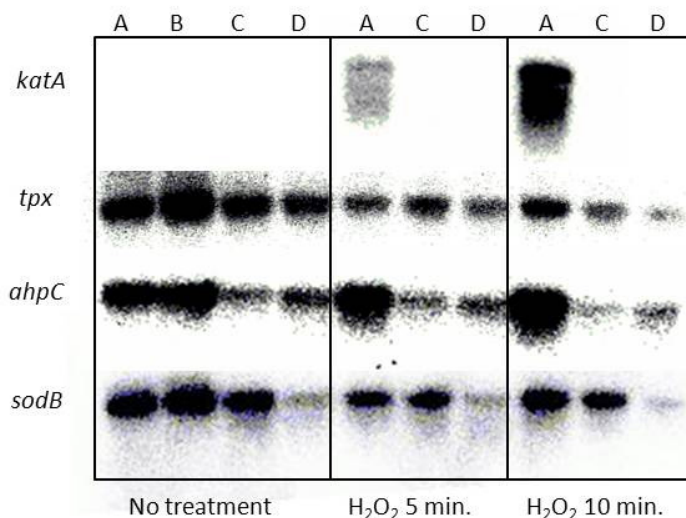
mutant is observed compared to the wild type, but the protein level in the same sample is decreased.

Opposite to the situation in the exponential phase, in the stationary phase a slight induction in the *dps* transcript level is observed when the wild type culture is pretreated with iron compared to iron-replete conditions. When cultures in iron-rich conditions are stressed with hydrogen peroxide, a decrease in transcript level occurs in the wild type sample, which correlates with the transcript level decrease observed in stationary phase in our previous results under iron-rich conditions (Figure 2-5).

It needs to be noted that, as seen in Figure 2-7, transcript levels in all four samples from the combined treatment with iron and hydrogen peroxide seem lower than the rest of samples under other conditions. However, in another replicate from this experiment the *dps* transcript levels from the last four samples were not at a lower level than the other samples, apart from the wild type sample (not shown) and therefore it is difficult to conclude which result is the most reliable. Nevertheless, from the two replicates we can still conclude that a decrease in *dps* transcript level was observed in the wild type culture from iron-rich conditions after treatment with hydrogen peroxide and that *dps* expression was derepressed in the  $\Delta fur$  mutant.

### **2.3.6 *sodB* is differentially expressed in the absence of Dps in stationary phase**

In an attempt to characterize the oxidative stress response in exponential and stationary phases, we compared the transcript levels of four known oxidative stress genes in the wild type and the  $\Delta dps$  strain in different growth phases with and without stress using Northern blots. The genes tested for were *kata*, *tpx*, *ahpC* and *sodB*. Due to the fact that RNA from the  $\Delta dps$  mutant in the exponential phase was damaged under oxidative stress (Figure 2-3), this sample was not included in the Northern blot analysis.



**Figure 2-8. Comparison of transcripts of four oxidative stress genes from *C. jejuni* 11168 before and after oxidative stress.** Wild type exponential (exp.) phase (A),  $\Delta dps$  exp. phase (B), wild type stationary (st.) phase (C),  $\Delta dps$  st. phase (D). Two biological replicates were performed and the representative result is shown for each gene.

We found *kataA* to be upregulated in the wild type strain in exponential phase in response to  $H_2O_2$ , but not in stationary phase cells (Figure 2-8). More *ahpC* transcript is present in exponential phase compared to stationary phase and transcription of *ahpC* is slightly induced by hydrogen peroxide in exponential phase. The *tpx* and *sodB* transcript levels are slightly higher in the  $\Delta dps$  mutant in exponential phase than in the wild type and the transcript level in the wild type from exponential phase is initially reduced upon exposure to hydrogen peroxide; however, it seems to increase again after 10 minutes. No difference in the transcript levels of *ahpC* and *tpx* was observed between the wild type and the mutant in stationary phase. Interestingly, there was a difference in *sodB* transcript level between the wild type and the mutant in stationary phase. Under conditions examined here, *sodB* is downregulated in the absence of Dps, regardless of whether the cells are stressed by hydrogen peroxide or not. All in all, the comparison of transcripts of selected oxidative stress genes under conditions examined here revealed that the differences between expression of most of these genes are growth phase dependent.

## 2.4 Discussion

*Campylobacter jejuni*, as many other pathogens, is well adapted to cope with the consequences arising from exposure to oxidative stress. An array of proteins the bacterium synthesizes includes among others KatA, AhpC, SodB or Tpx and they help to cope with deleterious effects of



accumulation of ROS. The data collected in this and other studies add up to show that Dps also belongs to this group of proteins important for the oxidative stress response. But whereas proteins such as catalase (KatA) act by converting harmful  $\text{H}_2\text{O}_2$  into water and oxygen (Grant and Park 1995), Dps prevents formation of toxic hydroxyl radicals by storing iron (Ishikawa et al. 2003), thereby not allowing the Fenton reaction to occur.

In this study we discovered that exponential phase cells of *C. jejuni* were more sensitive to peroxide stress than the stationary phase cells (Figure 2-1A). The lethal effect of hydrogen peroxide was concentration dependent and at higher concentrations the cells lacking Dps were also more sensitive to hydrogen peroxide than wild type cells in stationary phase, as presented in another study (Ishikawa et al. 2003). Treatment with 2.5 mM  $\text{H}_2\text{O}_2$  concentration caused detrimental damage to the exponential phase cells lacking Dps as seen by disintegration of DNA as well as RNA (Figure 2-2), (Figure 2-3). It is noteworthy that the DNA degradation could be either due to the accumulation of toxic hydroxyl radicals caused by lack of Dps, or could indicate that Dps binding to the DNA is necessary for its protection during stress in this growth phase. However, DNA binding ability of Dps has not been shown in *C. jejuni* so far, using *in vitro* binding assays (Ishikawa et al. 2003).

The Dps-dependent survival seen after treatment with hydrogen peroxide in exponential phase was not observed when the cells were treated with another oxidative agent, cumene hydroperoxide (Figure 2-1B). In another study it was found that *C. jejuni* cells lacking the AhpC protein were more sensitive to cumene hydroperoxide and atmospheric oxygen, but not hydrogen peroxide when examined in a disc inhibition assay (Baillon et al. 1999). This confirms that AhpC has its specificity directed towards organic peroxides, while Dps responds specifically to hydrogen peroxide. The fact that both the wild type and the  $\Delta dps$  mutant were equally sensitive to this oxidant in the exponential phase, but not the stationary phase, underlines the difference between the growth phases and shows that the exponential phase is more sensitive to the damage caused by this oxidant.

It is known that Dps production and requirement in other bacteria is growth-phase dependent and tightly regulated (Ingmer 2010). In a previous study, the level of Dps protein was found to be unchanged during growth between 9 and 72 hours and not induced by hydrogen peroxide (Ishikawa et al. 2003). In our study, we actually observed a twofold increase in the Dps protein level from exponential (4h) to stationary phase (24h) under no stress conditions, as well as a 1.4-fold induction of the protein in exponential phase upon exposure to hydrogen peroxide (Figure 2-5). Interestingly, exposure of the stationary phase cells to hydrogen peroxide caused

reduction in the Dps protein level as well as the *dps* transcript level. The fact that under no stress conditions high level of *dps* transcript in exponential phase results in low protein level and that in stationary phase culture lower *dps* transcript level results in increased protein level could suggest that Dps might be regulated at the post-transcriptional or post-translational level. Alternatively, increased protein level in the stationary phase compared to exponential phase could simply be due to accumulation of the Dps protein in the stationary phase where new *dps* transcript might no longer be produced. When comparing the levels of *dps* transcript and the Dps protein between the wild type culture and the strains deficient in regulators PerR and Fur in the stationary phase, we also observed a large increase in *dps* transcript in the  $\Delta fur$  mutant, while the protein level remained unchanged or slightly repressed compared to the wild type level (Figure 2-6), (Figure 2-7). This observation was an additional implication for post transcriptional or post translational regulation of Dps.

An example of such post-transcriptional regulation was observed in *Helicobacter pylori*, where protein NapA, a Dps homologue, was found to be regulated by CsrA, the carbon storage regulator (Barnard et al. 2004). The lack of CsrA caused deregulation of oxidative stress induced response of not only NapA, but also AhpC as well as many other important genes. It was observed that even though the *napA* transcript level was elevated in the *csrA* mutant, the NapA protein level was greatly reduced. This indicated that CsrA could be necessary for activation of translation of *napA* transcript. Given the high homology of *C. jejuni* with *H. pylori* (55.4% homologous genes, (Parkhill et al. 2000) and the presence of CsrA in *C. jejuni* genome it is likely that CsrA might be involved in Dps regulation, but additional experiments are needed to confirm it. A *C. jejuni csrA* mutant was already found to be required for survival under oxidative stress caused by both atmospheric oxygen and hydrogen peroxide (Fields and Thompson 2008). Several other forms of post transcriptional and post translational regulation of Dps homologues have been reported. In *E. coli*, the Dps protein was found to be abundant and stable during starvation and oxidative stress, however, after supplementing the medium with glucose, rapid ClpXP-mediated proteolysis of Dps was observed (Stephani et al. 2003). At the same time, *clpP* and *clpA* were also involved in maintaining Dps synthesis during starvation, indicating a large degree of post translational control. It is possible that the Clp proteolytic complex also plays a role in regulation of the amount of Dps in *C. jejuni*, as proteomic analysis of a  $\Delta clpP$  mutant of *C. jejuni* 11168 showed approximately 1.5-fold induction of the Dps protein in the absence of ClpP (Cohn et al. 2007). Interestingly, in another study *C. jejuni* Dps was, along with SodB and Tpx, reported to be one of the major phosphorylated proteins in 24 hour old cultures of *C. jejuni* 11168 (Voisin et al. 2007). In *Salmonella enterica*

serovar Typhimurium the Dps protein was found to be glycosylated with mannose, glucose and an unknown sugar residue (Hanna et al. 2007). The fact that the glycosylated form of Dps was detected at the highest level during the transition from exponential to stationary growth phase underlines that the function of this protein differs depending on the growth phases. Up to date, Dps glycosylation has not been reported in other microorganisms.

Knowing that iron regulates PerR and Fur (van Vliet et al. 1998; van Vliet et al. 1999) in *C. jejuni*, we investigated how Dps transcript changes in response to iron in *C. jejuni* strains lacking *perR*, *fur* or both genes and discovered that there are significant differences in regulation of *dps* depending on the growth phase. Based on our results we can conclude that in exponential phase both PerR and Fur act as repressors of *dps* and the similarity between the binding sites of these two repressors leads to speculation that they could compete for the binding site upstream of *dps*. Interestingly, while Fur remained a repressor in stationary phase, PerR became an activator of Dps in the absence of Fur, as no additional *dps* transcript induction was observed in the double mutant compared to the  $\Delta fur$  mutant (Figure 2-7). Additionally, we observed that changes in *dps* transcript level were dependent on iron during peroxide stress and that this dependence differed between growth phases. In exponential phase, the *dps* transcript level was induced under oxidative stress only in iron-rich conditions and not in iron-limited conditions, whereas in stationary phase oxidative stress caused decrease in *dps* transcript level in iron-rich conditions, but not iron-limited conditions. We also observed that in exponential phase the *dps* transcript level was increased in iron-limited conditions compared to iron-rich conditions in the wild type, which is in agreement with a previous microarray study, where transcription of *dps* was found to be 2.5 fold increased in iron-limited conditions (Holmes et al. 2005).

The comparison of transcription of other oxidative stress genes in the wild type and the *dps* mutant in exponential and stationary growth phases, including *kata*, *ahpC*, *sodB* and *tpx* confirmed that the stress response differs between these two growth phases. The *ahpC* and *kata* genes had higher transcription level in exponential phase than in stationary phase, which is in agreement with the published microarray study (Palyada et al. 2009) and one could speculate that the cells in exponential phase are either more vulnerable to oxidative stress in this growth phase, or that due to their rapid growth they are more ready and capable of eliciting a rapid response to changing stress conditions than the stationary phase cells. Of all the genes investigated, only *sodB* had a changed expression when Dps was missing in stationary phase. This observation could indicate that regulation of *sodB* expression is somehow linked to Dps or that the downregulation of *sodB* is caused by hydroxyl radicals that accumulate in the mutant, even under no stress conditions. In fact,

very recently, a new oxidative stress response regulator in *C. jejuni*, CosR, specific to  $\epsilon$ -proteobacteria, was revealed as a repressor of both Dps and SodB as well as other proteins including Rrc or LuxS and at the same time as a positive regulator of AhpC (Hwang et al. 2011). The downregulation of *sodB* observed in this study in the  $\Delta$ *dps* mutant was likely due to a co-regulation of these two genes by a common regulator. The genes *dps*, *sodB*, *katA*, *ahpC* and *perR* were also identified as members of the regulon of a novel oxidative stress regulator in *C. jejuni*, annotated *cj1556* (Gundogdu et al. 2011). While *sodB* gene was upregulated in the absence of the Cj1556 regulator, the other above mentioned genes, including *dps*, were downregulated.

Results presented in this study reveal that the mechanism and regulation of the function of Dps protein from *C. jejuni* are quite complicated and certainly more experiments are required to fully understand the interplay between Dps, its regulators PerR, Fur, CosR and Cj1556 and potentially other proteins involved in oxidative stress response, as well as to reveal mechanisms that control this protein in different growth phases. We showed that the response to oxidative stress in *C. jejuni* is growth phase dependent, as some of the oxidative stress genes had changed expression in that growth phase compared to the stationary phase and we observed that either post transcriptional or post translational regulation of Dps occurs to a greater extent in stationary phase.

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## **Chapter 3**

### **Manuscript II**

**Effect of chicken meat environment on gene  
expression of *Campylobacter jejuni* and its  
relevance to survival in food**

**Effect of chicken meat environment on gene expression of *Campylobacter jejuni* and its relevance to survival in food**

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## Abstract

Poultry meat is the major food source responsible for gastrointestinal infections caused by the human pathogen *Campylobacter jejuni*. Even though *C. jejuni* does not grow below 30°C, the bacterium survives on raw meat surfaces at refrigerated temperatures and thus poses a risk to the consumer. Previously, we have shown that chicken meat juice prolongs survival of *C. jejuni* at 5°C compared to laboratory medium, suggesting that compounds present in meat juice influence adaptation to low temperatures. In the present study we have used chicken meat juice to identify *C. jejuni* genes that are differentially expressed in a typical chicken meat environment encountered by consumers. The analysis showed that chicken meat juice increased expression of *luxS* involved in quorum sensing, as well as a gene involved in *O*-linked flagellin glycosylation in *C. jejuni*, while expression of haemin-uptake and the peroxide stress response genes were reduced. Furthermore, we propose that LuxS may play a key role in adaptation to the chicken meat juice environment, as lack of the *luxS* gene reduces the ability of *C. jejuni* to survive in chicken meat juice at low temperature. Finally, our data suggest that part of an ABC transport system is induced and we speculate that uptake of cryoprotectants may be important for *C. jejuni* to adapt to low temperature. In summary, we found that *C. jejuni* has a specific but limited transcriptional response to chicken meat juice and that *luxS* has an impact on the prolonged survival of *C. jejuni* in this important environment in the food chain.

Keywords: *Campylobacter jejuni*, food models, gene expression

### 3.1 Introduction

*Campylobacter jejuni* is a human pathogenic bacterium that can be found on poultry meat products in most Western countries. In the UK alone, up to 80% of raw retail chickens are contaminated with *Campylobacter* at levels of several thousands per cm<sup>2</sup> (Corry and Atabay 2001) and similar numbers were observed on chicken products in the Netherlands and the USA (Cason et al. 1997; Jacobs-Reitsma et al. 1995). *C. jejuni* are found in abundance in the cecum of chickens as commensal bacteria and during slaughter there is ample opportunity for *C. jejuni* to disseminate on processed meat and skin (Corry and Atabay 2001). Thus, *C. jejuni* is often present on poultry meat and causes the majority of bacterial gastrointestinal infections by consumption of undercooked poultry meat or by ingestion of other food items that have been cross-contaminated with *C. jejuni* originating from poultry meat products.

In contrast to other food borne pathogens, *C. jejuni* does not multiply in poultry meat products stored at refrigerated temperatures due to the minimum required temperature of 30°C for growth (Park 2002). However, several studies show that *C. jejuni* is able to survive on raw chicken meat and skin at refrigerated (4°C) and freezing (-20°C) temperatures for more than 10 days (Davis and Conner 2007; El-Shibiny et al. 2009). Thus, *C. jejuni* survives on refrigerated poultry meat products and poses a risk to the consumer, since ingestion of only 500 to 800 *C. jejuni* cells may cause illness (Black et al. 1988; Robinson 1981). Interestingly, *C. jejuni* does not encode an orthologue of the cold shock protein CspA (Parkhill et al. 2000), which functions as RNA chaperone that allows efficient translation at low temperature (Yamanaka et al. 1998). However, several studies suggest that *C. jejuni* actually responds and adapts to low temperature. First of all, *C. jejuni* still produces ATP and consumes oxygen at 4°C, indicating that vital cellular processes are functioning at low temperature (Hazeleger et al. 1998) and secondly *C. jejuni* responds to cold shock by changing gene expression (Stintzi and Whitworth 2003). These results suggest that an adaptive response to low temperature occurs in laboratory media; however, no studies aimed at understanding how a food environment affects the adaptive mechanism have been conducted. We have previously developed a chicken meat juice model that can be used to study *C. jejuni* under conditions that mimic the environment the bacterium encounters on poultry meat products (Birk et al. 2004). Interestingly, we found that *C. jejuni* survives for a significantly longer time in chicken meat juice at low temperatures compared to laboratory growth medium (Birk et al. 2004). During long term storage at 5°C, the colony forming units per ml in the chicken meat juice was maintained above 10<sup>7</sup> for 30 days, whereas the colony forming units per ml in BHI started to decrease already after 12 days (Birk et al. 2004). We speculate that compounds present in the chicken meat juice



may affect *C. jejuni* physiology, and that adaptation to the chicken meat juice environment involves changes in gene expression. In the present work we have investigated this hypothesis by comparing genome-wide gene expression of *C. jejuni* NCTC11168 in BHI growth medium and chicken meat juice at 5°C.

This work is a part of the EU funded Biotracer project that focuses on tracking and tracing of pathogenic microorganisms in food and feed chains. An essential part of this project is devoted to obtaining biological knowledge about the behaviour of the pathogens under conditions they might encounter on their route from the source to the consumer. In this study we investigate how *C. jejuni* reacts to chicken meat juice at low temperature and identify the genes that specifically change expression as a response to this environment. Knowledge of specific processes that allow *C. jejuni* to adapt to the chicken meat environment may be used to develop efficient hurdle technologies such as ingredients for marinating or specific packaging material or processes resulting in safer food.

## **3.2 Materials and methods**

### **3.2.1 Bacterial strains and growth conditions.**

*C. jejuni* NCTC11168 was supplied from the National Collection of Type Cultures and *C. jejuni* NCTC11168H *luxS::kan* mutant was kindly donated by B. Bourke (Corcionivoschi et al. 2009). *C. jejuni* strains were routinely grown on Blood Agar Base II (Oxoid) supplemented with 5% calf blood (Base II) or in Brain Heart Infusion broth (BHI, Difco) at 42°C in a microaerobic environment (6% O<sub>2</sub> and 6% CO<sub>2</sub>) inside a Forma Series II Water Jacketed CO<sub>2</sub> Incubator (Thermo Electron Corporation). When appropriate, media were supplemented with kanamycin (50 µg/ml).

### **3.2.2 Isolation of a *C. jejuni* NCTC11168 *luxS* mutant.**

Natural transformation was used to introduce a *luxS::kan* mutation into *C. jejuni* NCTC11168 using essentially the method described before (Wang and Taylor 1990). After plating on Basell plates containing kanamycin and incubation for 3 days in microaerobic atmosphere we isolated several *C. jejuni* NCTC11168 *luxS::kan* mutants.

### **3.2.3 Preparation of chicken meat juice.**

Chicken juice was prepared as described by Birk et al. (2004). Commercially available frozen chickens without giblets were thawed overnight in a container at 4°C. The chicken juice was then collected, centrifuged to remove larger particles, sterile-filtered and stored at -20°C. Before the day of the experiment, chicken juice was thawed overnight at 4°C.

### **3.2.4 Collection of samples for total RNA extraction.**

*C. jejuni* NCTC11168 was grown at 42°C under microaerobic conditions to mid-exponential phase ( $OD_{600}=0.5$ ) and 40 ml of the culture was harvested at 8228 x *g* for 5 minutes at 5°C. The pellet was resuspended in 1.6 ml cold (5°C) BHI, yielding a 25 x concentrated culture, which was then incubated for 2 h at 4°C to allow adaptation to atmospheric conditions as well as low temperature. Subsequently, aliquots of 0.8 ml of the concentrated culture were transferred to tubes containing 6.7 ml 5°C chicken meat juice or BHI. Immediately after that, 2 ml from either chicken meat juice or BHI were dispensed into 4 ml RNA protect reagent (Qiagen) and these samples were denoted “time point 0 minutes”. The remaining 5 ml of either chicken meat juice or BHI containing bacteria was divided into two cooled tubes and incubated at 5°C for 5 and 30 minutes, respectively and 2 ml of the cultures were then transferred into 4 ml RNA protect reagent. In all cases bacteria were harvested at 8228 x *g* for 2 minutes at 0°C, the supernatant was decanted and the remaining pellets were stored at -20°C until RNA extraction.

### **3.2.5 Total RNA extraction.**

The total RNA was extracted using the RNeasy Mini Kit (Qiagen), with slightly modified protocol, essentially as described before (Moen et al. 2005). During the extraction all samples were treated with 10 µg DNaseI for 15 minutes at room temperature. The concentrations of the RNA samples were measured using Nanodrop 1000 spectrophotometer. All samples were run on gel and checked for purity and lack of degradation using 2100 Bioanalyzer (Agilent Technologies). In addition, several samples were tested for the presence of genomic DNA by PCR.

### **3.2.6 Microarrays and data analysis.**

*C. jejuni* NCTC11168 whole-genome microarrays (Jones et al. 2004) were supplied by the Bacterial Microarray Group at St. Georges's (BµG@S [<http://www.bugs.sghms.ac.uk>]). RNA vs DNA common reference microarrays were performed as described previously (Sidders et al. 2007). Briefly, 1 µg of *C. jejuni* 11168 gDNA was labelled with Cy5 using Klenow (large fragment) polymerase (Invitrogen) and 5 µg of mRNA with Cy3 using reverse transcriptase (Invitrogen). Purified Cy3/Cy5 labelled cDNA was incubated on the microarray, underneath a LifterSlip (Eerie scientific), at 65°C for 16-20 hours. The microarrays were scanned using GMS418 Microarray Scanner (Genetic Microsystems). Fluorescence was quantified with ImaGene software v5.5 (BioDiscovery) and initially processed using MAVI Pro v2.6.0 (MWG Biotech) and analyzed using GeneSpring v7.3.1 software (Agilent

Technologies). Data from triplicate biological experiments were combined and significant differential gene regulation was identified by ANOVA (Welch t-test) with a p-value cut-off 0.05.

### **3.2.7 Testing survival of *luxS::kan* mutant in chicken juice at low temperature.**

*C. jejuni* NCTC11168 and NCTC11168 *luxS::kan* were grown in BHI at microaerobic conditions at 42°C until OD<sub>600</sub>=0.5 was reached. The bacteria were harvested at 8228 x *g* for 5 minutes at 5°C and subsequently resuspended in 1.6 ml of BHI at temperature of 5°C. The cultures were then left in aerobic atmosphere at 5°C for 2 hours. Next, 0.8 ml of each culture was transferred into tubes with 6.7 ml of BHI and 6.7 ml chicken juice, yielding 4 tubes in total. Immediately after placing the cultures in the media, CFU per ml was determined. Cultures were incubated aerobically at 5°C and CFU per ml was determined twice a week for a period of 30 days.

## **3.3 Results and discussion**

### **3.3.1 Genome-wide gene expression in chicken meat juice**

In this study we investigated the adaptive response of *C. jejuni* to chicken meat juice at 5°C, which has been shown to lead to prolonged survival (Birk et al. 2004). To identify differential responses in gene expression in chicken meat juice and BHI, pre-adapted cells of *C. jejuni* NCTC11168 were inoculated into chicken meat juice or BHI and RNA was extracted after 5 and 30 minutes. Microarray analysis of triplicate biological replicates identified five genes  $\geq$  2-fold upregulated and four genes  $\geq$  2-fold downregulated (Table 1). The experiment was designed to eliminate stress arising from both oxygen and low temperature and the changes that we see are specifically related to the adaptation to the chicken meat juice environment. Thus, as seen in Table 1 there were only few significant changes in the gene expression of *C. jejuni* at low temperature in the chicken meat juice compared to BHI.

### **3.3.2 LuxS is important for adaptation to low temperature in chicken meat juice**

The *luxS* gene was identified by the microarray to be upregulated in *C. jejuni* after 30 minutes in chicken meat juice (Table 3-1). *luxS* encodes S-ribosylhomocysteinase (LuxS), which catalyzes the formation of autoinducer-2 (AI-2) molecules and homo-cysteine. The latter is a component of the activated methyl cycle that provides S-adenosyl-L-methionine for methylation of RNA, DNA and protein. AI-2 production is common among both Gram-positive and Gram-negative pathogenic bacteria and AI-2 functions as a molecule for interspecies communication, also known as quorum sensing (Sun et al. 2004). It has been shown that AI-2 production is not only regulated by cell

density, but also in response to nutritional and environmental conditions, for example the presence of certain carbohydrates in the medium (Surette and Bassler 1999). Furthermore, AI-2 activity could be detected in *C. jejuni* after growth at 37°C for 24 h in milk, chicken broth or Brucella broth, while little or no AI-2 activity could be found in the same media after incubation at 4°C for 3 h (Cloak et al. 2002). Thus, these data suggest that the AI-2 production may not be very prominent at low temperature. Yet, the induction of the *luxS* gene indicates that the quorum sensing system is activated in the chicken meat juice and may thus be important for switching to an adaptive response in this particular environment.

Several studies have revealed important functions of LuxS in *C. jejuni* and characterization of *luxS* deficient strains have shown that AI-2 or the activated methyl cycle may play a role in motility, autoagglutination, biofilm formation, oxidative stress, host colonization and interaction with epithelial cells (Elvers and Park 2002)(He et al. 2008;Jeon et al. 2003)(Reeser et al. 2007); (Quinones et al. 2009). To investigate whether *luxS* is important for adaptation of *C. jejuni* to low temperature in chicken meat juice, we compared survival of a *C. jejuni luxS* mutant with the parental strain. We found that after six days of incubation at 5°C, the viable counts of the *luxS* mutant started to decrease in the chicken meat juice, whereas the wild type viable counts remained practically unchanged for 27 days (Figure 3-1A). Furthermore, survival of *C. jejuni* at low temperature was less dependent on *luxS* in BHI as the difference between the parental strain and the mutant is less pronounced in this media (Figure 3-1B). This supports our notion that *luxS* is important for *C. jejuni* in adaption to low temperature in chicken meat juice.

### 3.3.3 Cytolethal distending toxin

Interestingly, lack of the *luxS* gene was previously found to reduce transcription of the *cdtABC* genes by two fold (Jeon et al. 2005). The *cdtABC* genes encode the cytolethal distending toxin subunits A, B and C in *C. jejuni*, which act by blocking the G<sub>2</sub>/M phase of eukaryotic cells prior to cell division, leading to cytoplasmic distension and cell death (Whitehouse et al. 1998). In the present study, we found that the *cdtA* gene was upregulated to approximately the same level as *luxS* (Table 3-1). The physiological explanation for upregulation of this toxin in chicken meat juice is not clear; however the upregulation may simply be due to increased transcription from *luxS* gene.

### 3.3.4 Transport of iron

Within five minutes after *C. jejuni* was exposed to the chicken meat juice, a two-fold downregulation of *chuD* gene was observed (Table 3-1). *chuD* encodes a periplasmic binding protein that is a part of

a haemin-uptake system. Transcriptional downregulation of *chuD* indicates that *C. jejuni* does not lack iron when incubated in chicken juice and therefore, increased haemin uptake is not necessary. However, another gene putatively involved in iron transport (*cfbpC*) was two-fold upregulated after 30 minutes in chicken juice (Table 3-1). CfbpC contains two domains found in ABC transport systems; spermidine/putrescine/amino acid uptake (COG3842) and carbohydrate/solute uptake (cd3259). Since uptake of cryoprotectants such as polyamines (spermidine and putrescine) and polar amino acids have been described as protecting cell viability at low temperature (Yancey et al. 1982) we speculate that increased uptake of cryoprotectants from the chicken meat juice may contribute to adaptation of *C. jejuni* to low temperature.

### **3.3.5 O-linked flagellin glycosylation**

Gene *cj1323*, encoding a likely glycosyl transferase, was found to be increasingly upregulated over time, showing 2-fold upregulation after 5 minutes, and almost 4-fold upregulation after 30 minutes (Table 3-1). *cj1323* is part of the *C. jejuni* O-linked flagellin glycosylation locus, responsible for post-translational modification of the flagella subunits and is important for flagella assembly as well as flagella secretion (Karlyshev et al. 2005). Recently, *cj1323* and adjacent genes have been implicated in the addition of a legionaminic acid modification of the flagellin (Howard et al. 2009). *cj1323* is found in approximately 50% of *C. jejuni* strains and is particularly associated with isolates from chickens and other livestock (Champion et al. 2005). It is possible that *C. jejuni* strains that have *cj1323* and associated genes are more adapted to chicken and the food chain.

### **3.3.6 Other genes have altered expression in chicken juice**

Another gene that was downregulated after 5 minutes exposure to chicken meat juice was a probable integral membrane protein of unknown function (*cj0560*) (Table 3-1). Two genes were two-fold downregulated within 30 minutes after exposure: *cj1712* that showed 40.9% identity to *Helicobacter pylori* 81168 HP1530 (purine nucleoside phosphorylase (*punB*)) and *ahpC*, encoding alkyl hydroperoxide reductase, which is known to have a role in peroxide stress (Baillon et al. 1999). Noteworthy, *ahpC* was found to be upregulated in a *C. jejuni luxS* mutant (He et al. 2008), suggesting that the downregulation may be mediated by LuxS.

### 3.4 Concluding remarks

In the present study we have identified genes that alter their expression after exposing *C. jejuni* to chicken meat juice at low temperature. Previously, the *in vitro* cold shock response of *C. jejuni* was investigated (Stintzi and Whitworth 2003), however, in our study only genes that specifically responded to the chilled chicken meat juice environment were identified. The analysis revealed only a limited number of genes with changed expression and showed that chicken meat juice increased expression of *luxS* involved in quorum sensing and the activated methyl cycle as well as a gene involved in *O*-linked flagellin glycosylation in *C. jejuni*, while expression of haemin-uptake and the peroxide stress response genes were reduced. Interestingly, the upregulation of *luxS* may affect peroxide stress, *O*-linked flagellin glycosylation and cytolethal distending toxin synthesis, as supported by genome-wide transcriptional analysis of a *luxS* mutant (He et al. 2008). Interestingly, addition of exogenously produced AI-2 does not induce changes in gene expression, hence suggesting that the metabolic function of LuxS rather than quorum sensing results in the observed transcriptional changes (Holmes et al. 2009). The loss of viability of the *luxS* mutant confirms that LuxS plays a key role in adaptation to the chicken meat juice environment at low temperature. Finally, our data suggest that an uptake system was induced that may contribute to survival of *C. jejuni* in chicken meat juice. In summary, we found that *C. jejuni* has a specific, but limited transcriptional response to chicken meat juice and future studies will show if physiological changes, such as increased uptake of cryoprotectants, may have an impact on the prolonged survival of *C. jejuni* in chicken meat juice.

### Acknowledgments

The *C. jejuni* NCTC11168H *luxS* mutant received from B. Bourke, UCD, Dublin, Ireland was constructed by A.V. Karlyshev, LSHTM, UK as described (Corcionivoschi et al. 2009). We sincerely appreciate the expert technical assistance of Christel Galschiøt Buerholt. This study was financially supported by the European Union funded Integrated Project BIOTRACER (contract FOOD-2006-CT-036272) under the 6th RTD Framework.

### Figure legends

**Figure 3-1. Survival of *C. jejuni* NCTC11168 (wild type) and *luxS* mutant in chicken meat juice at 5°C.** *C. jejuni* wild type (circles) and *luxS::kan* mutant (triangles) cultures were incubated both in A) chicken meat juice (CJ, empty symbols) and B) BHI (Brain Heart Infusion, filled symbols) broth at 5°C for a period of 29 days. Samples were taken out twice a week in order to determine CFU/ml. One representative of three experiments is shown and similar results were obtained in all three experiments.

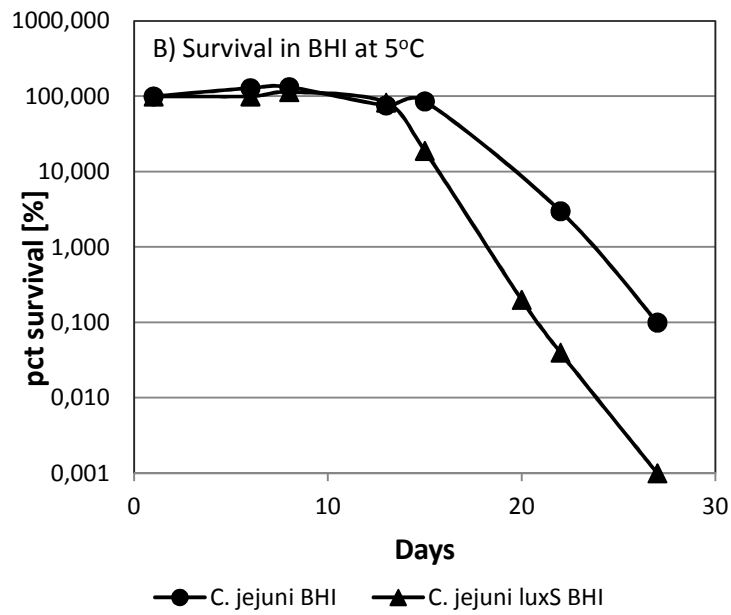
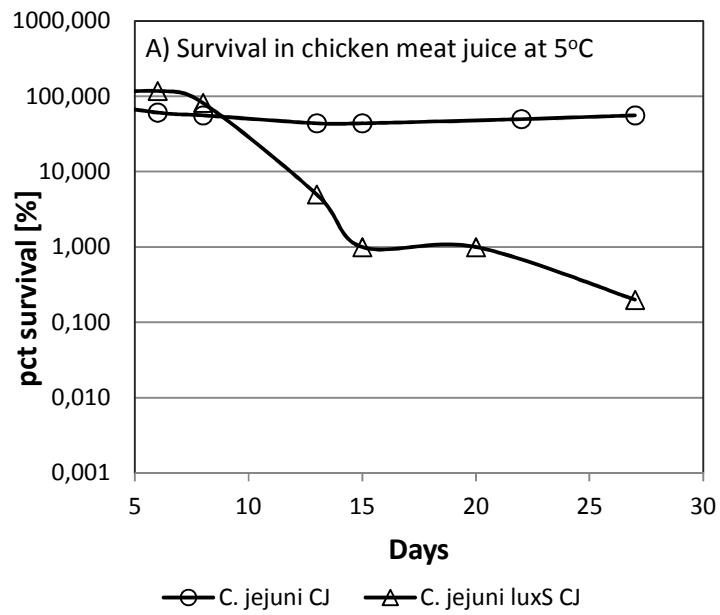
**Table 3-1. Microarray analysis of gene expression in *C. jejuni* NCTC11168 in chicken juice model compared to BHI at 5°C.**

Gene name	Normalized ratio <sup>a</sup> 5 min	p-value <sup>b</sup>	Normalized ratio <sup>a</sup> 30 min	p-value <sup>b</sup>	Product
<i>cj0079c</i> ( <i>cdtA</i> )	1.9	0.001	<b>2.1</b>	0.033	Cytolethal distending toxin
<i>cj0173c</i> ( <i>cfbpC</i> )	1.6	0.135	<b>2.0</b>	0.014	Putative iron-uptake ABC transporter (ATP binding)
<i>cj0334</i> ( <i>ahpC</i> )	0.6	0.142	<b>0.5</b>	0.036	Alkyl hydroperoxide reductase
<i>cj0560</i>	<b>0.5</b>	0.006	0.9	0.790	Probable integral membrane protein
<i>cj1198</i> ( <i>luxS</i> )	1.1	0.434	<b>2.0</b>	0.043	S-ribosyl-homocysteinase
<i>cj1323</i>	<b>2.1</b>	0.065	<b>3.7</b>	0.006	Part of O-linked glycosylation locus
<i>cj1617</i> ( <i>chuD</i> )	<b>0.5</b>	0.043	0.6	0.493	Putative haemin uptake system periplasmic haemin-binding protein
<i>cj1712</i>	1.2	0.644	<b>0.5</b>	0.010	40.9% identity to HP1530 (purine nucleoside phosphorylase ( <i>punB</i> ))

<sup>a</sup> Normalized ratio indicates the ratio of fluorescent between test and control. Genes with a normalized ratio  $\leq 0.5$  or  $\geq 2$  at either 5 or 30 min are shown in bold.

<sup>b</sup>P-value calculated using ANOVA (Welsh t-test,  $p=0.05$ ) in GeneSpring v7.3.1.





## **Chapter 4**

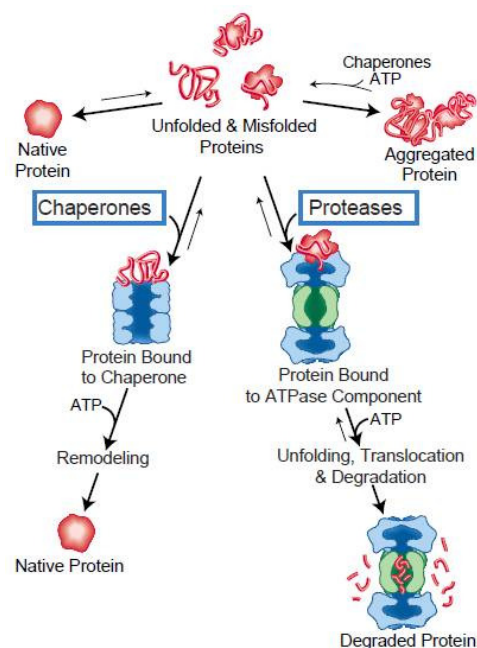
### **Supplementary results I**

#### **The role of the Clp proteolytic complex in *C. jejuni* stress response**

## 4.1 Introduction

Bacterial pathogens, such as *Campylobacter jejuni*, are often exposed to stress conditions both inside the host as well as in the environment. Encountered stress has many adverse effects on the bacteria and one of them is protein misfolding and subsequent aggregation (Tyedmers et al. 2010; Winkler et al. 2010).

Chaperones and proteases are common in every type of organism and together they create a large superfamily of AAA+ proteins (ATPases Associated with diverse cellular Activities) that can circumvent misfolded protein accumulation either by degradation or refolding of misfolded proteins (Snider et al. 2008). One of these important protein families is the Clp family and it includes both Clp proteins that are exclusively chaperones, as well as ATPases that have a chaperone activity themselves, but can also associate with a proteolytic subunit to act as an ATP-dependent protease (Gottesman et al. 1997). Both chaperones and ATP-dependent proteases play a crucial role in preventing accumulation of misfolded proteins which is illustrated in Figure 4-1 as a so-called protein triage model (Wickner et al. 1999).



**Figure 4-1. Protein triage model (Wickner et al. 1999).** Nonnative proteins that are recognized by chaperones are remodeled in an attempt to reach a native conformation. If that succeeds, the native protein is removed from the triage model. If remodeling fails, the nonnative protein is released by the chaperone and it will most likely be degraded when it encounters a protease. Proteins that cannot be remodeled or degraded will form aggregates with other nonnative proteins.

From Figure 4-1 it is visible that if both chaperone and proteases fail to remodel or degrade nonnative proteins, protein aggregation will occur. In fact, mutations in either proteases or chaperones lead to increased accumulation of misfolded proteins, decreased viability and make the cells more sensitive to different types of stresses, such as heat shock (Baek et al. 2011;Cohn et al. 2007;Frees and Ingmer 1999;Thomsen et al. 2002;Wickner et al. 1999).

The Clp proteolytic complex is an ATP-dependent protease that consists of a Clp proteolytic subunit (ClpP) which may interact with different Clp ATPases and together they play an important role in the cell by degrading misfolded proteins, thereby preventing their aggregation (Wickner et al. 1999). Clp complexes are widely distributed in different organisms and summary of the different protease-chaperone complexes and their functions in prokaryotes is presented in Table 4-1.

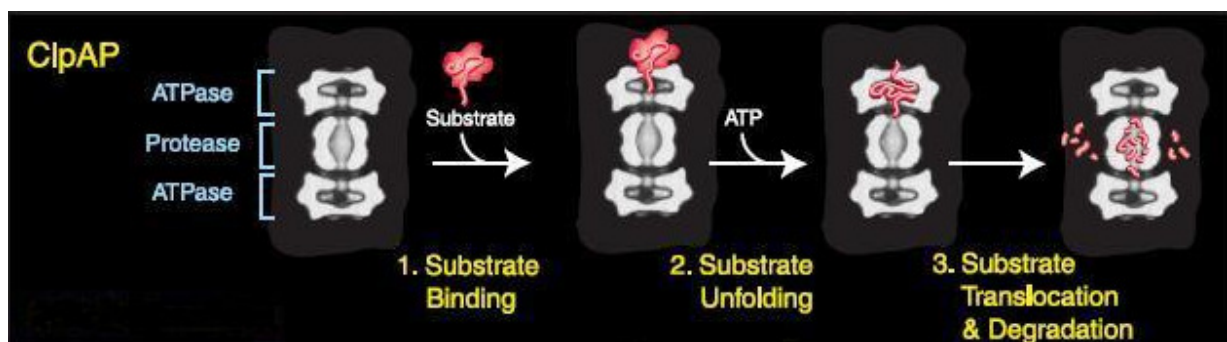
**Table 4-1. Occurrence and function of Clp chaperone-protease complexes in prokaryotes (modified from (Kress et al. 2009))**

Chaperone-protease complex	Occurrence	Cellular function	References
Class I			
ClpAP	<i>Proteobacteria</i>	Protein quality control	(Zolkiewski 2006)
ClpCP	<i>Firmicutes, Actinobacteria, Cyanobacteria</i>	Competence development and sporulation, transcription and regulation of stress response	(Turgay et al. 1998) (Kruger et al. 2001)
ClpEP	<i>Firmicutes</i>	Thermotolerance, cell division and virulence	(Miethke et al. 2006) (Nair et al. 1999)
Class II			
ClpXP	<i>Proteobacteria, Firmicutes, Actinobacteria, Cyanobacteria, Deinococcus-Thermus, Fusobacteria, Spirochaetes, Aquificae, Thermatogae</i>	Protein quality control, cell division, transposition, virulence	(Zolkiewski 2006) (Camberg et al. 2011) (Jones et al. 1998) (Frees et al. 2003)
ClpYQ	<i>Proteobacteria, Firmicutes, Spirochaetes, Aquificae, Thermatogae</i>	Cell division, heat shock response and capsule transcription	(Wu et al. 1999) (Chuang et al. 1993)

The ClpXP complex is the one most widely spread among prokaryotes (Table 4-1). ClpA is more common in Gram negative proteobacteria, whereas ClpC and ClpE are found in Gram positive

bacteria and cyanobacteria (ClpC only). ClpYQ is present in most Gram negative as well as some Gram positive bacteria, usually acting alongside ClpAP.

The ClpP protease subunit consists of two stacked heptameric rings of identical subunits that together create an internal chamber with 14 proteolytic sites (Figure 4-2) (Wickner et al. 1999) . The Clp ATPase subunits, whether it is ClpA or ClpX, can associate on both sides of the proteolytic subunit, thereby creating an entry point for the substrates. The ATPases recognize, bind and partially unfold the substrate and then help translocate it into the proteolytic chamber, in an ATP-dependent process. Inside the proteolytic chamber, degradation of the unfolded substrate into smaller peptides occurs without the use of ATP. Some ATPases, like ClpX in *E. coli*, are also capable of acting as chaperones independently of their protease partner (Jones et al. 1998).



**Figure 4-2. ATP-dependent proteolysis (modified from Wickner *et al.* 1999).** Schematic model of steps in protein degradation by the Clp proteolytic complex in prokaryotes. *E. coli* ClpAP is presented as an example.

The ATPases are capable of recognizing their substrates themselves, however, the substrate specificity of ClpA in *E. coli* was additionally found to be modulated by an adapter protein ClpS, which modifies the substrate recognition of ClpA by binding to its N-terminal domain (Dougan et al. 2002). A number of other adapter proteins have been identified in *E. coli* and *B. subtilis* (Kirstein et al. 2009).

The role of the Clp proteolytic complexes has been studied in a number of bacteria and below, several examples are presented. In a Gram negative pathogen, *Salmonella enterica* serovar Typhimurium, a *clpP* mutant had reduced ability to grow at high temperatures, low pH and high salt concentration (Thomsen et al. 2002). Apart from that, the *clpP* mutant could not degrade the abnormal puromycyl-containing peptides as efficiently as the wild type, which proves that ClpP is required for degrading misfolded proteins arising under stress conditions. In the Gram positive bacterium *Lactococcus lactis* the ClpP protease was found to be required for tolerance to both heat

and the tRNA analogue puromycin, which causes formation of truncated and misfolded proteins (Frees and Ingmer 1999). The *L. lactis* ClpX ATPase was found to be not only involved in heat shock response, but also in cryoprotection (Skinner and Trempey 2001). In another study focusing on *Staphylococcus aureus* (Frees et al. 2003), the absence of ClpX or ClpP not only reduced transcription of the *agr* effector molecule RNAlII, but also reduced the activity of autoinducing peptide (AIP). In *S. aureus*, the *agr* locus is required for regulation of the *hla* gene, which produces  $\alpha$ -hemolysin, an extracellular protein necessary for the bacterium's pathogenicity (Vandenesch et al. 1991).

In addition to the role of ATP dependent proteases in degradation of non-native proteins, the Clp proteolytic complexes were also shown to play an important role in various regulatory functions (Weichart et al. 2003). For example, the ClpXP complex was found to be involved in regulation of *E. coli* starvation sigma factor ( $\sigma^S$ ) (Schweder et al. 1996). The sigma factor is known to be induced upon entry into stationary phase in the wild type *E. coli* cells and it regulates a number of genes that lead to increased stress resistance of the cells (Lange and Hengge-Aronis 1994). ClpXP was found to degrade the  $\sigma^S$  factor in the exponential phase but not in stationary phase, allowing the accumulation of the factor and thereby upregulation of genes associated with stationary phase (Schweder et al. 1996). In another study it was shown that at least one of the ClpP, ClpA and ClpX proteins is involved in control of the important growth-phase dependent genes in *E. coli* (Weichart et al. 2003). In *Salmonella enterica* serovar Typhimurium ClpXP was found to be a negative regulator for the flagellum synthesis, as a strain lacking ClpXP exhibited an overproduction of flagella (Tomoyasu et al. 2002). These studies are only several examples showing that the Clp proteolytic complexes have significance not only for stress tolerance, but also for regulation of virulence and other cellular functions.

In *Campylobacter jejuni*, the ClpXP proteolytic complex is responsible for degradation of heat-damaged proteins (Cohn et al. 2007). The mutants lacking either *clpP* or *clpX* were unable to grow at 42°C and 44°C, with the former being the temperature of the intestinal tract of poultry, whereas the cells lacking *clpA* grew at these temperatures just as well as the wild type.

This suggests not only a role of the ClpXP complex in degrading heat-damaged proteins in *C. jejuni*, but it could also mean that the complex could have importance in the ability to colonize the chicken intestine. Furthermore, the *clpP* mutant had reduced motility and autoagglutination, as well as reduced ability to invade INT407 epithelial cells. Since all these phenotypes are related to virulence (Golden and Acheson 2002; Misawa and Blaser 2000), these data suggested that the ClpP protease might also be required for virulence of *C. jejuni*. With these results in mind, I aimed to

further investigate the role of the ClpAP and ClpXP complexes in chicken colonization, adaptation to stationary phase as well as stress tolerance. The latter two conditions are of special relevance to those experienced by the bacterium in a slaughter house, where *C. jejuni* will be exposed to fluctuations in temperatures, oxygen concentrations and nutrient limiting conditions (Corry and Atabay 2001).

## 4.2 Materials and methods

### 4.2.1 Bacterial strains and growth conditions, plasmids and primers used in this study

The bacterial strains and plasmids are listed in Table 4-2 and Table 4-3. All *C. jejuni* strains were grown at 37°C in microaerobic conditions (88% N<sub>2</sub>, 6% O<sub>2</sub> and 6% CO<sub>2</sub>) in BHI broth (Brain Heart Infusion, company) or cultivated on Base II agar plates with 5% bovine blood. Ampicillin (100 µg/ml), Chloramphenicol (20 µg/ml), Kanamycin (50 µg/ml) and Streptomycin (50 µg/ml) were added to the media for growth of appropriate mutant strains. *E. coli* was cultivated in Lysogeny broth or agar and incubated at 37°C.

**Table 4-2. Strains used in this study.**

Strain	Characteristics	Reference
<i>C. jejuni</i> LB1238	<i>rpsL</i> <sup>*</sup> , Strep <sup>R</sup>	Baek et al. 2011
<i>C. jejuni</i> ML1.1 – ML1.6	<i>clpX::cat-rpsL</i>	This study
<i>C. jejuni</i> ML2.1 – ML2.2	<i>clpP::cat-rpsL</i>	This study
<i>C. jejuni</i> ML3.1 – ML3.6	<i>clpA::cat-rpsL</i>	This study
<i>C. jejuni</i> LB1263	<i>clpX::cat</i>	Cohn et al 2007
<i>C. jejuni</i> LB1277	<i>clpP::cat</i>	Cohn et al 2007
<i>C. jejuni</i> MTA11	<i>clpA::cat</i>	Cohn et al 2007
<i>C. jejuni</i> MTA24	<i>clpS::cat</i>	Marianne Thorup Cohn, unpublished
<i>C. jejuni</i> ML260	<i>clpP::cat + clpP</i>	This study
<i>C. jejuni</i> ML261	<i>clpP::cat + clpP</i>	This study
<i>C. jejuni</i> ML263	<i>clpP::cat + clpP</i>	This study
<i>E. coli</i> AMS6P	$\Delta clpP$ , Cam <sup>R</sup>	Schweder et al. 1996
<i>E. coli</i> DH5 $\alpha$	F <sup>-</sup> $\phi 80lacZ\Delta M15 \Delta(lacZY A-argF)U169 deoR recA1 endA1 hsdR17(r_k^-, m_k^+) phoA supE44 thi-1 gyrA96 relA1 \lambda^-$	Invitrogen

**Table 4-3. Plasmids used in this study.**

Plasmid	Characteristics	Reference
pLB248	pGEM7:: $\Delta clpX::cat rpsL$ , Cam <sup>R</sup>	Lone Brøndsted, unpublished
pLB254	pGEM7:: $\Delta clpP::cat rpsL$ , Cam <sup>R</sup>	Lone Brøndsted, unpublished
pmta42	$\Delta clpA::cat rpsL$ , Cam <sup>R</sup>	Marianne Thorup Cohn, unpublished
pmta43	$\Delta clpS::cat rpsL$ , Cam <sup>R</sup>	Marianne Thorup Cohn, unpublished
pLB222	pGEM7:: $\Delta clpP$ , Amp <sup>R</sup>	Cohn et al. 2007
pLB223	pGEM7:: $\Delta clpX$ , Amp <sup>R</sup>	Cohn et al. 2007
pmta35	$\Delta clpA$ , Kan <sup>R</sup> , Amp <sup>R</sup>	Marianne Thorup Cohn, unpublished
pmta37	$\Delta clpS$ , Kan <sup>R</sup> , Amp <sup>R</sup>	Marianne Thorup Cohn, unpublished
pKfdxA	$\Delta cj0046$ , Kan <sup>R</sup>	Duncan Gaskin, unpublished

**Table 4-4. Primers used in this study**

Primer name	Sequence
clpP-for	5'-ATCGTCTCACATGTTTATTCCTTATGTT-3' *
clpP-rev	5'-ATCGTCTCACATGTCATTTAAACTTTT-3'
clpX-for	5'-ATCGTCTCACGTGGAATTCCTGTCTATA-3'
clpX-rev	5'-ATCGTCTCACATGTTAACTTATTTTTCT-3'
clpA-for	5'-ATCGTCTCACATGAAATACCAAGAAAAT-3'
clpA-rev	5'-ATCGTCTCACATGTTAGAATATAAATTC-3'
clpS-for	5'-ATCGTCTCACATGCCAAAAACCCAAACT-3'
clpS-rev	5'-ATCGTCTCACATGTCATTCTTCTTCCAC-3'
ClpA-A	5'-CCAACAGAACTCATGCAAGAAG-3'
ClpA-D	5'-GCTTACCATGCTTTCACCAAAG-3'
KB2F	5'-CCCTGCACATATAGTATGAC-3'
KB2R	5'-GCAAGTCTTGTAATTCATCC-3'
KB6F	5'-TTCAATTCCGGTGATATTCTCATTTTAGCC-3'
KB6R	5'-ATGTTTATTACAGCTACAGCTCCAAATCC-3'

\*BmsBI restriction site in bold

#### 4.2.2 Construction of clean deletion mutants

A previously published method of defined deletion mutagenesis, based on Streptomycin counter-selection, was used (Hendrixson et al. 2001; Skorupski and Taylor 1996). The method is based on the fact that mutations in the *rpsL* gene, encoding 30S ribosomal protein S12, confer Streptomycin resistance to the strain and that these mutations are recessive in a strain expressing the wild type protein (LEDERBERG 1951). Briefly, in this two-step method, illustrated in Figure 4-3, a large region of the gene of interest in a background strain with Streptomycin resistance (*rpsL*<sup>\*</sup>) is replaced with a counterselectable cassette containing a *cat* gene conferring Chloramphenicol



resistance and *rpsL* gene expressing the wild type ribosomal protein S12 in the first step. In this way, the resulting strain is sensitive to Streptomycin and resistant to Chloramphenicol. In the second step, the cassette is replaced with the deleted gene of interest and the strain regains Streptomycin resistance while losing Chloramphenicol resistance.

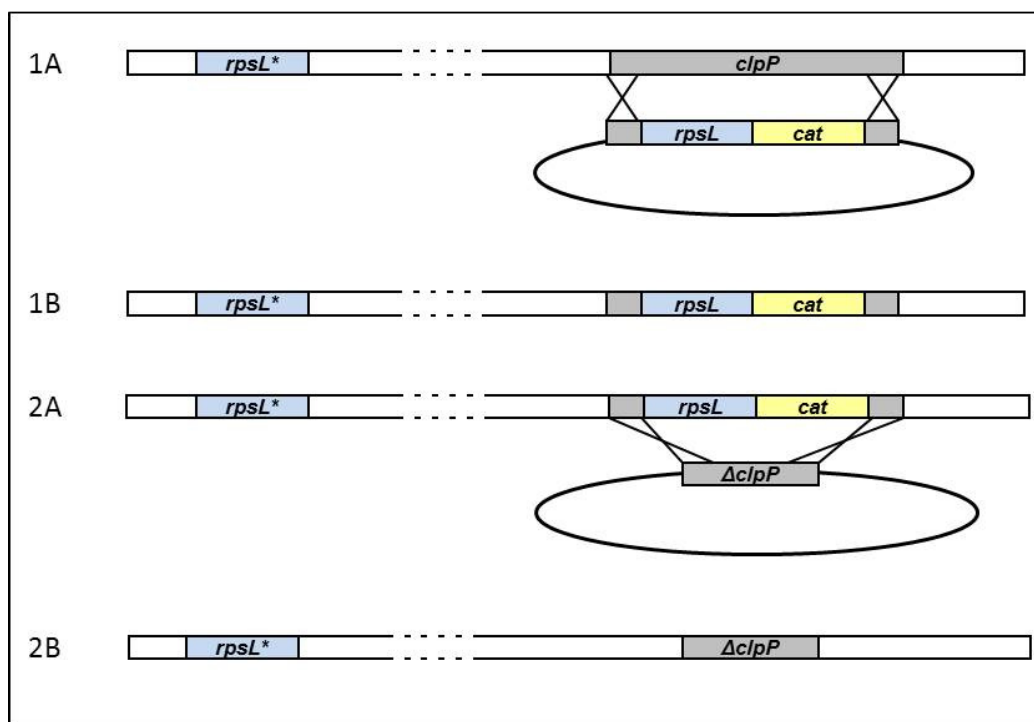
A spontaneous Streptomycin resistant mutant of *C. jejuni* NCTC11168 was isolated by streaking the strain on plates containing Streptomycin. Then the mutated *rpsL* gene (800 bp), known to confer Streptomycin resistance, was amplified by PCR, cloned to a pCR2.1 vector (Invitrogen) and transferred into *C. jejuni* NCTC11168, resulting in strain LB1238 (*rpsL*<sup>Sm</sup>) (Baek et al. 2011). This step was performed in order to ensure that the Streptomycin resistance was only a result of mutation in the *rpsL* gene and not elsewhere on the chromosome.

To construct clean deletion mutants in the *clpP*, *clpA*, *clpX* and *clpS* genes, strain LB1238 was transformed by electroporation with plasmid constructs containing genes of interest disrupted by a counterselectable cassette *cat-rpsL* (Table 4-3 and Table 4-5) and selected for Chloramphenicol resistance to confirm that the wild type *clp* gene was replaced with the disrupted genes by homologous recombination (step 1A in Figure 4-3).

**Table 4-5. Deletions of the *clp* genes in the plasmid constructs used to make clean deletion mutants**

Gene (bp)	Bp upstream on vector	Bp downstream on vector	Bp deleted
<i>clpP</i> (585)	833 (43 bp of <i>clpP</i> )	778 (59 bp of <i>clpP</i> )	483 bp
<i>clpA</i> (2130)	436 (99 bp of <i>clpA</i> )	537 (119 bp of <i>clpA</i> )	1912 bp
<i>clpX</i> (1254)	756 (15 bp of <i>clpX</i> )	801 (59 bp of <i>clpX</i> )	1180 bp
<i>clpS</i> (291)	399 (29 bp of <i>clpS</i> )	401 (53 bp of <i>clpS</i> )	209 bp

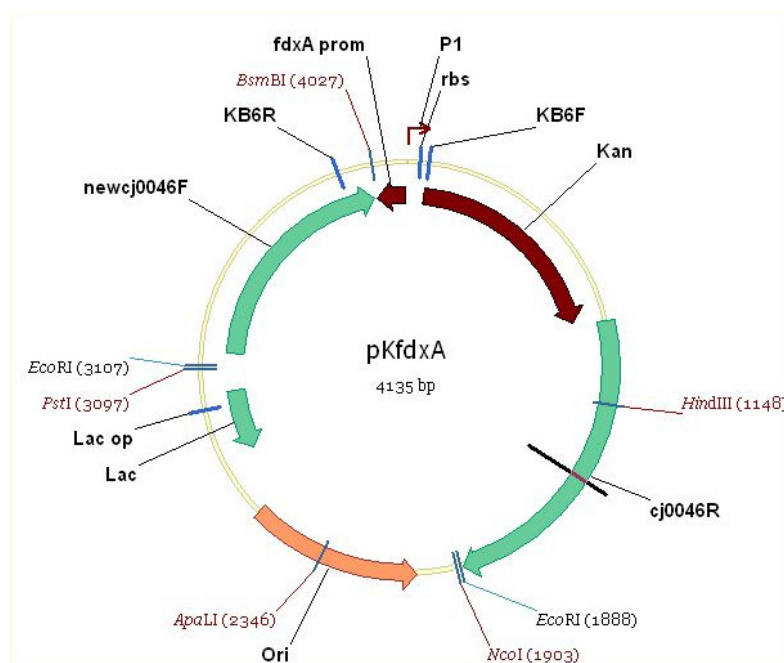
After 4 days of incubation several single transformants were isolated for each of the constructs and were subsequently scored for Streptomycin sensitivity. At this step the *rpsL* mutation in LB1238 conferring Streptomycin resistance is still present on the chromosome in these strains; however, it is a recessive mutation and since the resulting strains also have the counterselectable cassette *cat-rpsL* inserted, they are Strep<sup>S</sup> and Cam<sup>R</sup> (step 1B in Figure 4-3). When possible, the presence of the *cat-rpsL* cassette on the chromosome was verified by colony PCR. Then the *cat-rpsL* cassette was replaced with a deleted gene by transforming the intermediate strain with plasmid constructs containing deletions of each *clp* gene (step 2A in Figure 4-3) and selecting for Streptomycin resistance (step 2B in Figure 4-3). Subsequently, colonies were screened for loss of Chloramphenicol resistance.



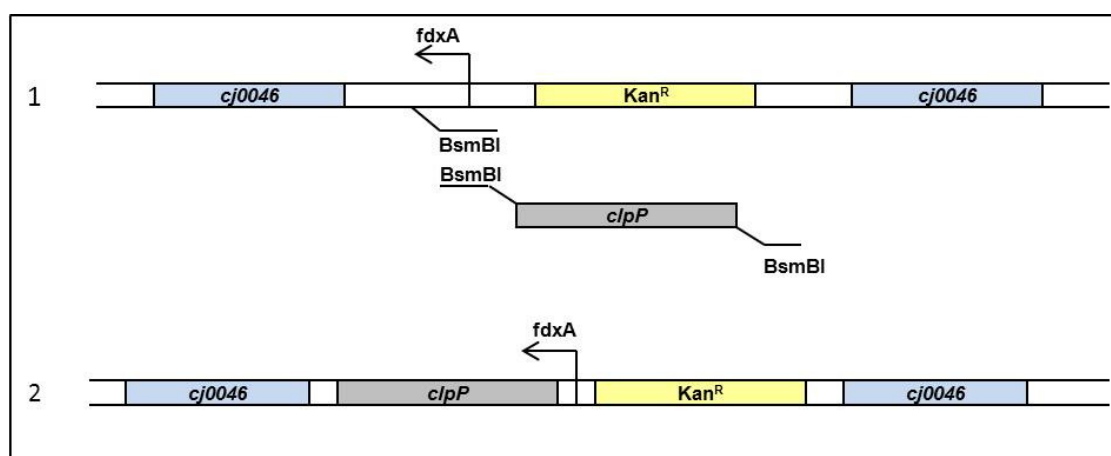
**Figure 4-3. Strategy for constructing clean deletion mutants.** *C. jejuni* LB1238 (*Strep<sup>R</sup>, Cam<sup>S</sup>*) was transformed with plasmids containing each one of the four genes (*clpP* given as an example), disrupted with an *cat-rpsL* cassette (1A), resulting in a strain with a phenotype *Strep<sup>S</sup>, Cam<sup>R</sup>* (1B). That strain was then transformed with plasmids containing clean deletions of each gene (2A), resulting in a strain with a phenotype *Strep<sup>R</sup>, Cam<sup>S</sup>* (2B).

#### 4.2.3 Construction of strains complementing mutations

The pKfdxA vector, as well as the protocol for the gene complementation used in this study was kindly donated by Dr Duncan Gaskin. Each of the four *clp* genes (*clpP*, *clpA*, *clpX* and *clpS*) was amplified with primers containing BsmBI restriction sites (Table 4-4). The resulting PCR products and the pKfdxA vector (Figure 4-4) were then cut with the Esp3I enzyme (an isoschizomer of BsmBI, Fermentas) and ligated, placing the gene under the control of the *fdxA* promoter (Figure 4-5).



**Figure 4-4. Map of the pKfdxA vector.** Image created in Vector NTI, Invitrogen



**Figure 4-5. Example of cloning strategy leading to obtaining complementation mutants.** Each of the four genes was inserted into the unique BsmBI site on the vector (1), placing the gene under the control of the fdxA promoter (2).

Ligated constructs were sequenced to determine the presence of the insert, its correct orientation as well as lack of point mutations. Vectors with successfully cloned genes were then introduced by electroporation into the relevant mutant strains with the respective *clp* genes disrupted by the *cat* gene and the transformants were selected for Chloramphenicol and Kanamycin resistance.

#### **4.2.4 Phenotype testing of the complemented strains by comparing growth ability at different temperatures (adapted from Cohn et al. 2007)**

The lawns of *C. jejuni* NCTC11168 (ML200), *C. jejuni*  $\Delta clpP$  (LB1277) and *C. jejuni*  $\Delta clpP + clpP$  (ML260, ML261, ML263) were grown overnight in microaerobic conditions at 37°C. The lawns of bacteria were then harvested with 1 ml BHI and the OD<sub>600</sub> was adjusted to 0.1 in 1 ml. Six 10-fold dilutions of the strains were made in FKP (physiological salt solution with peptone) and 3 x 10 µl of each dilution were spotted on appropriate Basell agar plates. Three plates with each strain were placed in three different temperatures: 37°C, 42°C and 44°C and incubated for 3 days in microaerobic conditions (CampyGen, Oxoid). The growth of the strains at different temperatures was then compared.

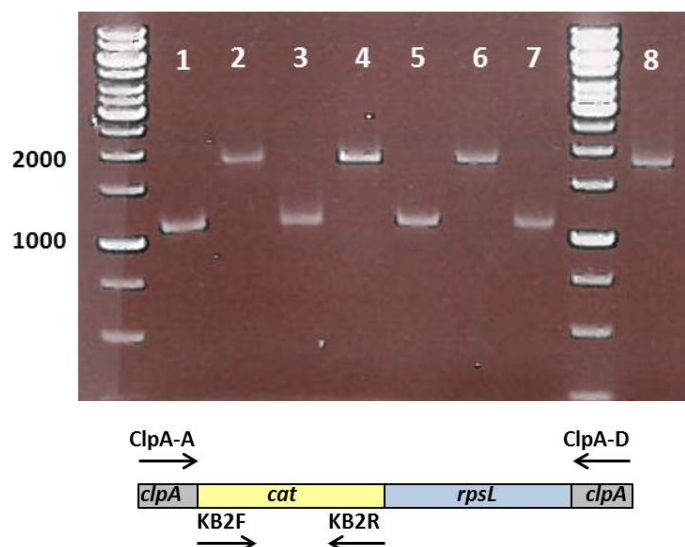
### **4.3 Results and discussion**

#### **4.3.1 Clean deletion mutants**

One of the mutants used in the study by Cohn et al. (2007), the  $\Delta clpP$  mutant, was shown to have polar effect on the *def* gene located downstream due to insertion of the antibiotic resistance cassette into the *clpP* gene. Therefore we decided to construct clean deletion mutants in genes *clpP*, *clpA* and *clpX*, where each *clp* gene would be removed by a clean in-frame deletion. We also decided to include the *clpS* gene in our study as the role of this gene has not been investigated in *C. jejuni*.

A number of Chloramphenicol resistant transformants were isolated after the introduction of *clpP*, *clpX* and *clpA* deletions carrying the *cat-rpsL* cassette (step 1B in Figure 4-3), while it was not possible to obtain any transformants from the construct containing the *clpS* gene for unknown reasons.

Insertions in the *clpP*, *clpA* and *clpX* genes were confirmed by PCR using two primer pairs amplifying regions upstream and downstream of the insertion site and containing parts of the *cat-rpsL* cassette (Figure 4-6), as the products had the expected size. It also needs to be noted that some colonies were still present when these strains were restreaked on Streptomycin plates.

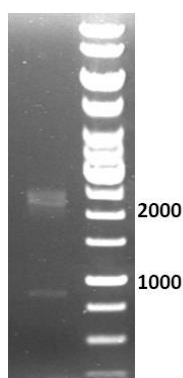


**Figure 4-6. Colony PCR of several first-step transformants containing the *clpA* construct.** PCR products from two primer pairs are shown for each colony. Primer locations are illustrated. Lanes 1, 3, 5, 7: primer pair ClpA-A and KB2R, expected size: 1049 bp, Lanes 2, 4, 6, 8: primer pair KB2F- ClpA-D, expected size: 1790 bp.

In the second part of the method (steps 2A and 2B in Figure 4-3) we tried to replace the *cat-rpsL* cassettes in *clpP*, *clpX*, and *clpA* genes with the fragment carrying clean deletion of the respective *clp* gene by selection for Streptomycin resistant transformants. Subsequently, approximately 1200 colonies were screened for each of the three constructs and surprisingly, all of them were resistant to Chloramphenicol in addition to Streptomycin, which suggested that the *cat-rpsL* cassette was still present in the chromosome and that the Streptomycin counterselection did not work.

Another observation made while constructing the mutants was that when the second-step transformants (step 2B in Figure 4-3) were restreaked on Chloramphenicol and Streptomycin, only few single colonies were visible on Chloramphenicol plates after one day of incubation, but on the second day of incubation the growth of these strains on Chloramphenicol plates was just as good as on Streptomycin. More transformations were performed (step 2A in Figure 4-3) using increasing Streptomycin concentrations in order to increase the selection for Streptomycin and thereby to make sure that the *cat-rpsL* cassette was crossed out of the chromosome. Twenty two transformants were found that were sensitive to Chloramphenicol, however, the phenotype was not stable and only after one more day of incubation the transformants were resistant to Chloramphenicol again. Provided that the method of Streptomycin counterselection works properly, the situation where the resulting strain is resistant to both antibiotics should never occur.

PCR performed on genomic DNA from one of the second-step *clpA* mutants revealed that the cell material, from which the gDNA was purified, probably contained two types of cells; some with a cassette and others without, since the larger band corresponds to the size of the *clpA* gene containing the *cat-rpsL* cassette and the smaller band corresponds to the size of the deleted *clpA* gene (Figure 4-7). It should be noted here that the genomic DNA used in this PCR was not purified from a liquid culture grown from a single colony, but rather from a scrape of cells, for technical reasons.



**Figure 4-7. Amplification of *clpA* region of one of the second-step mutants.** The bigger band corresponds to 2300 bp, size of *clpA* with the *cat-rpsL* cassette and the other band, 800 bp, to size of *clpA* without the cassette.

Streptomycin counterselection was also developed for *Helicobacter pylori* (Fischer et al. 1999). However, the method turned out to be difficult to apply, since very few of the Strep<sup>R</sup> transformants obtained in the last step sustained the desired gene replacement (Dailidienė et al. 2006). The suspected reason for that was frequent gene conversion between the homologous wild type *rpsL* and the mutated *rpsL* alleles used in *H. pylori* which resulted in the undesired Strep<sup>R</sup> transformants outnumbering the desired ones. The authors of that study overcame this problem by constructing their *rpsL,erm* cassette using the *rpsL* gene from *C. jejuni*, since it differs by 18% in DNA sequence from that of *H. pylori*, however the protein sequences encoded by the genes from the two pathogens are 95% similar. In this way a larger sequence divergence was introduced between the two versions of *rpsL*, which minimized the probability of gene conversion. As a result, two types of transformants were still obtained in the final step. One type was the desired transformant, which regained Streptomycin resistance and lost resistance to Erythromycin and the other type was a gene convertant that also had the Strep<sup>R</sup> phenotype, but retained Erythromycin resistance as a result of recombination between the two versions of *rpsL* gene (Dailidienė et al. 2006). The yield of the desired transformants among the Strep<sup>R</sup> colonies was between ~17% and ~90%, depending on

the locus and strain background used (several different ones were tested in this study), which made it quite easy to find the desired transformant. Therefore, the most likely reason for the Cam<sup>R</sup> phenotype in our case is genetic rearrangements that could have occurred between the wild type and the mutated *rpsL* gene, so that the resulting transformant would harbor the *cat* gene inserted into one of the *clp* genes, as well as the mutated *rpsL* gene on both locations on the chromosome.

Alternatively, one could speculate that a single crossover event had occurred in step 2A in Figure 4-3, either upstream or downstream of the *cat-rpsL* cassette, instead of double crossover. In that case, the whole vector would cross into the chromosome and PCR using primers complementary to the *clpA* gene would lead to two products of different sizes, as in Figure 4-7, as well as explain the Chloramphenicol resistance of the strain. This possibility could be easily checked for by plating the transformants on a medium containing antibiotics (Ampicillin or Kanamycin) to which the vectors are resistant (see Table 4-3).

Finally, it is worth noting that other groups reported using the same method without success. One of them aimed at deleting the lipooligosaccharide biosynthesis region in *C. jejuni* (Marsden et al. 2009). In another case, an in-frame deletion of the *spoT* gene in *C. jejuni* was attempted, but the intermediate strain was never obtained (Gaynor et al. 2005). The authors suspected that this was due to the polar effect on genes downstream of *spoT*, occurring after insertion of the *cat-rpsL* cassette. Perhaps constructing new vectors with a *cat-rpsL* cassette containing the *rpsL* gene from *H. pylori* would allow for minimized gene conversion and thereby increase the chances of obtaining the desired mutants in *C. jejuni*.

#### 4.3.2 Complementation mutants

Instead of making clean deletions in the *clp* genes we decided to complement the insertion mutants from the previous study (Cohn et al. 2007) with a wild type version of each *clp* gene inserted into a pseudogene elsewhere on the chromosome of *C. jejuni* 11168. Introducing a wild type *clp* gene that is constitutively expressed from another location on the chromosome into the mutant strain allows checking whether the phenotype of the complemented strain is the same as that of the wild type or not. If it is the same, it means that the mutation introduced into the gene of interest does not have a polar effect on the expression of the downstream genes. However, if the phenotype is different and especially if it is similar to the mutant phenotype, it could indicate that the mutation in the gene of interest is most likely not responsible for the change in the mutant phenotype compared to wild type. Instead, the reason could be the polar effect on a downstream gene caused by the mutation.

The strategy was first to clone each of the wild type *clp* genes into the vector pKfdxA (Figure 4-4). Here, the *clp* genes were inserted between two regions of a pseudogene, *cj0046*, and placed under control of a constitutive promoter (*fdxA*) (Figure 4-5). Next, each *clp* mutant strain was transformed with the corresponding vector and the wild type *clp* gene should be inserted into the bacterial chromosome by homologous recombination of the regions encoding the pseudogene on the vector with the corresponding regions on the chromosome. The pKfdxA vector has a Kanamycin resistance marker which was desired for selection since the mutant strains are resistant to Chloramphenicol.

The *clpA*, *clpX* and *clpS* genes were successfully inserted into the pKfdxA vector. However, it was not possible to obtain any transformants when the ligation mixture containing vector and the *clpP* gene was transformed into *E. coli* DH5 $\alpha$ . In order to minimize possible problems with proper digestion of the PCR products with the BsmBI enzyme, we attempted to modify the procedure by adding an additional step, where *clpP* gene was at first cloned into the TOPO vector, then cut out and ligated into the target vector, pKfdxA. However, no transformants were obtained from the ligation as well.

The most probable reason for the lack of transformants was constitutive expression of the *clpP* gene from the pKfdxA vector, which might have been toxic to the recipient cells already producing their own ClpP. To test this hypothesis, we transformed the ligation into a *clpP*-deficient *E. coli* strain (Schweder et al. 1996) and only then it was possible to obtain the desired transformants, which confirmed our explanation.

Even though the other genes were cloned successfully, sequencing revealed that point mutations were present in the cloned *clpA* and *clpX* genes, which have led to changes in the protein sequences they encoded (Figure 4-8 and Figure 4-9).



```

ClpA      MKYQENLQKYLDNAKNLSLINHHEFVTCEHVLFALLKLSTDFKDI FEEFSDGDLELLETE 60
Seq       MKYQENLQKYLDNAKNLSLINHHEFVTCEHVLFALLKLSTDFKDI FEEFSDGDLELLETE 60
          *****

ClpA      LKNYISQNNQVIKQEIEPTISVVLDEILLSSKNKNNEIKIIDFLEKLIQDSRSYSSYLLK 120
Seq       LKNYISQNNQVIKQEIEPTISVVLDEILLSSKNKNNEIKIIDFLEKLIQDSRSYSSYLLK 120
          *****

ClpA      KHNINLNKIQELQNHENIQNLNNHTSDLTLLAQNGKIDPLIGRKFELEMMQILSRKKKN 180
Seq       KHNINLNKIQELQSHENIQNLNNHTSDLTLLAQNGKIDPLIGRKFELEMMQILSRKKKN 180
          *****

ClpA      NPILVGEAGVGKTAIVEGLALAI AEKKVPKNLQNAKIFSLDMASILAGTKYRGDFEKRIK 240
Seq       NPILVGEAGVGKTAIVEGLALAI AEKKVPKNLQNAKIFSLDMASILAGTKYRGDFEKRIK 240
          *****

```

**Figure 4-8. Sequence alignment of the ClpA protein with the sequenced part from the vector.** Only parts of the sequences are shown. There was a point mutation at position 134, which has led to conversion of Asparagine (N) to Serine (S). Alignment was performed using ClustalW2 program.

```

ClpX      VEFLSIGVKIMPRKCSFCNEVENPQRRILANENDDAFICEYCVGAYSIIYGEEKEFKEP 60
Seq       VEFLSIGVKIMPRKCSFCNEVENPQRRILANENDDAFICEYCVGAYSIIYGEEKEFKEP 60
          *****

ClpX      KQSHNTEFKDITPKELKAYLDYVIGQDRAKKVFSVGVNHYKRLFKAEQDDDELFKS 120
Seq       KQSHNTEFKDITPKELKAYLDYVIGQDRAKKVFSVGVNHYKRLFKAEQDDDELFKS 120
          *****

ClpX      NILLVGPTGSGKTLLAQTLAKFLDVPIAICDATSLTEAGYVGEDVENILTRLLQAADGDV 180
Seq       NILLVGPTGSGKTLSAQTLAEFLDVPIAICDATSLTEAGYVGEDVENILTRLLQAADGDV 180
          *****

ClpX      QRAQKGIVFIDEIDKIARMSENRSITRDVSGEGVQQALLKIIEGSLVNIIPRGGGRKHPNQ 240
Seq       QRAQKGIVFIDEIDKIARMSENRSITRDVSGEGVQQALLKIIEGSLVNIIPRGGGRKHPNQ 240
          *****

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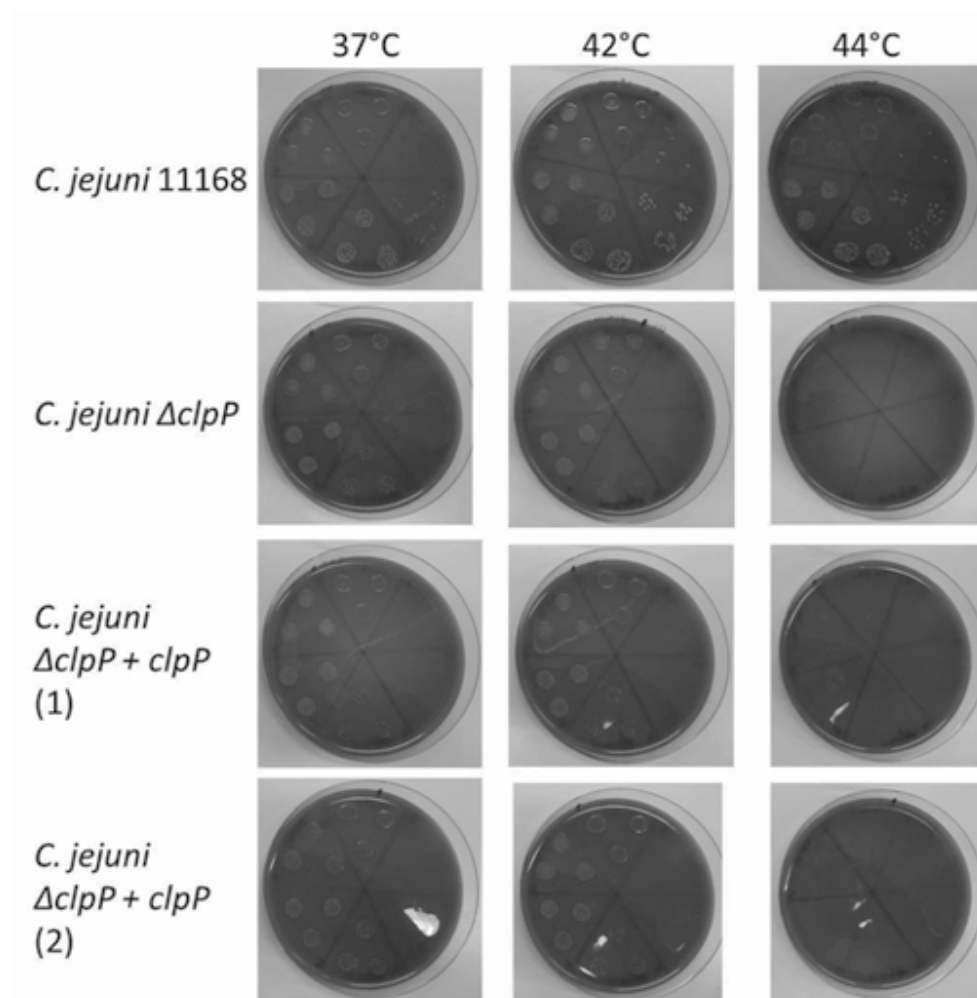
**Figure 4-9. Sequence alignment of the ClpX protein with the sequenced part from the vector.** Only parts of the sequences are shown. There were at least two point mutations at positions 135 and 141, which have led to conversion of Leucine (L) to Serine (S) and Lysine (K) to Glutamic acid (E). Alignment was performed using ClustalW2 program.

The *clpP* and *clpS* genes had no mutations that would affect the protein sequence. We decided to continue only with the *clpP* mutant, as originally we planned to investigate *clpS* together with the *clpA* mutant, for which it is known to be an adapter protein in *E. coli* (Dougan et al. 2002).

The vector containing the *clpP* gene was transformed into the appropriate strain (LB1277). Only transformants that were resistant to both Chloramphenicol (from the disrupted *clp* genes) and Kanamycin (from the vector fragment crossed into the pseudogene *cj0046*, see Figure 4-5) were chosen for further analysis. In order to check whether the complemented mutants were capable of complementing the mutations, we compared the growth of the wild type *C. jejuni*,  $\Delta clpP::cat$  mutant and  $\Delta clpP::cat + clpP$  complemented strain at 37°C, 42°C and 44°C, as described before (Cohn et al.

2007). Both the wild type strain and the complemented strain should be able to grow well at all three temperatures, whereas the  $\Delta clpP::cat$  mutant is known to grow very poor at 42°C and 44°C.

Unfortunately, after 2 days of incubation at these temperatures we could conclude that the phenotype of the complemented strain was exactly like the one of the mutant, that is, the  $\Delta clpP::cat + clpP$  strain did not grow well at 42°C and did not grow at all at 44°C. Three transformants with the *clpP* complemented mutation were tested in this way and all of them exhibited the same phenotype (two of the transformants tested are shown in Figure 4-10).



**Figure 4-10. Comparing phenotypes of the *C. jejuni* 11168,  $\Delta clpP$  mutant and  $\Delta clpP+clpP$  complemented mutant by testing the ability to grow at three different temperatures.** The same phenotype was observed for three different transformants containing the complemented *clpP* gene.

One could speculate that since the phenotype of the complemented strain is the same as in the mutant, the observed sensitivity of the complemented strain to 42°C and 44°C is a result of the

polar effect on the downstream *def* gene. The *def* gene encodes peptide deformylase and is located directly downstream of the *clpP* gene (Gundogdu et al. 2007; Parkhill et al. 2000). Even though it was shown to be an essential gene in *E. coli* (Mazel et al. 1994), the reduced level of expression from *def* in the *clpP* mutant reported in Cohn et al. (2007) did not affect the viability of the strain. Apart from that, 2D gel analysis verified the presence of both formylated and deformylated proteins, thereby confirming active deformylase activity. Therefore we believe that even though *def* expression is reduced in the *clpP* mutant, the gene is still functional and unlikely to be the cause of the observed mutant phenotype at higher temperatures. Furthermore, as described in Cohn et al. (2007), *clpX* but not *clpA* mutant shared the same phenotype with *clpP* mutant, indicating that both ClpX and ClpP, presumably as a complex, are responsible for degradation of heat-damaged proteins. All in all, we believe that the observed *clpP* mutant phenotype is not linked to the polar effect but is due to the lack of ClpP alone.

There could be several reasons for explaining the unexpected phenotype from the complemented *clpP* mutant. By selecting and propagating the strain on plates containing both Chloramphenicol and Kanamycin we confirmed that the part of the insert from the vector, conferring Kanamycin resistance (Figure 4-4) is functional in *C. jejuni* genome. Kanamycin expression is controlled by a separate promoter; hence we do not know whether the constitutive *fdxA* promoter controlling *clpP* expression is functional or not in *C. jejuni*. We suspect however that it was functional in *E. coli*, since it was not possible to obtain ligation products of the *clpP* gene and pK*fdxA* vector in *E. coli* DH5 $\alpha$ , but it was possible in the  $\Delta$ *clpP* *E. coli* strain. Assuming that it is functional, it would mean that the ClpP protein is being continuously produced in the complemented strain. Up to date, it is not known how the *clpP* gene is regulated in *C. jejuni*. Knowing that the ClpP protein is necessary for *C. jejuni* to survive at temperatures of 42°C and 44°C (Cohn et al. 2007), one could speculate that its production is induced at these temperatures in the wild type and perhaps it is only the wild type *clpP* promoter that can respond to this type of induction. In that situation increase in temperature would cause no ClpP induction in the complemented strain because the *fdxA* promoter would not respond to this stimulus and it is likely that the ClpP protein level constitutively produced from *fdxA* promoter is either too low or too high to allow growth of the complemented strain at elevated temperatures. It would be helpful to compare the amount of the ClpP protein in the wild type and in the complemented strain at different temperatures to check whether that is the case.

To sum up, we believe that the method of constructing the complemented *clpP* mutant was successfully conducted in our study; however, it was not possible to utilize it fully in the individual

case of *C. jejuni* ClpP protease for the reasons mentioned above. Further attempts to obtain mutation-free constructs containing genes encoding ATPase subunits ClpA and ClpX, as well as the adapter protein ClpS were not undertaken due to lack of time in the project.

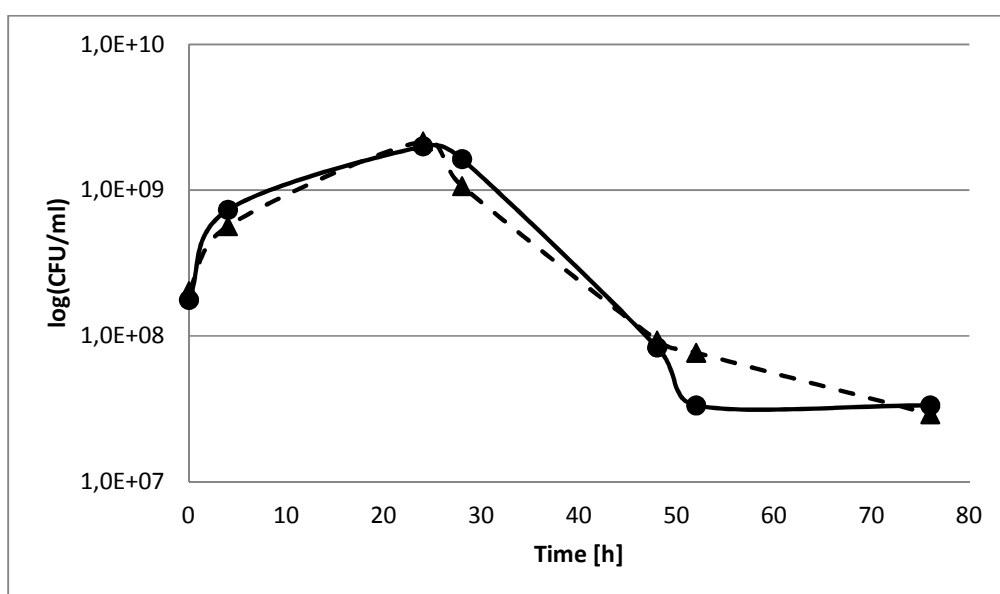
# **Chapter 5**

## **Supplementary results II**

This chapter contains additional results that were not used in any of the manuscripts. Each result is preceded with a short description of the methodology used and ends with the result description and conclusion.

## 5.1 Growth of *C. jejuni* wild type and $\Delta dps$ mutant strains

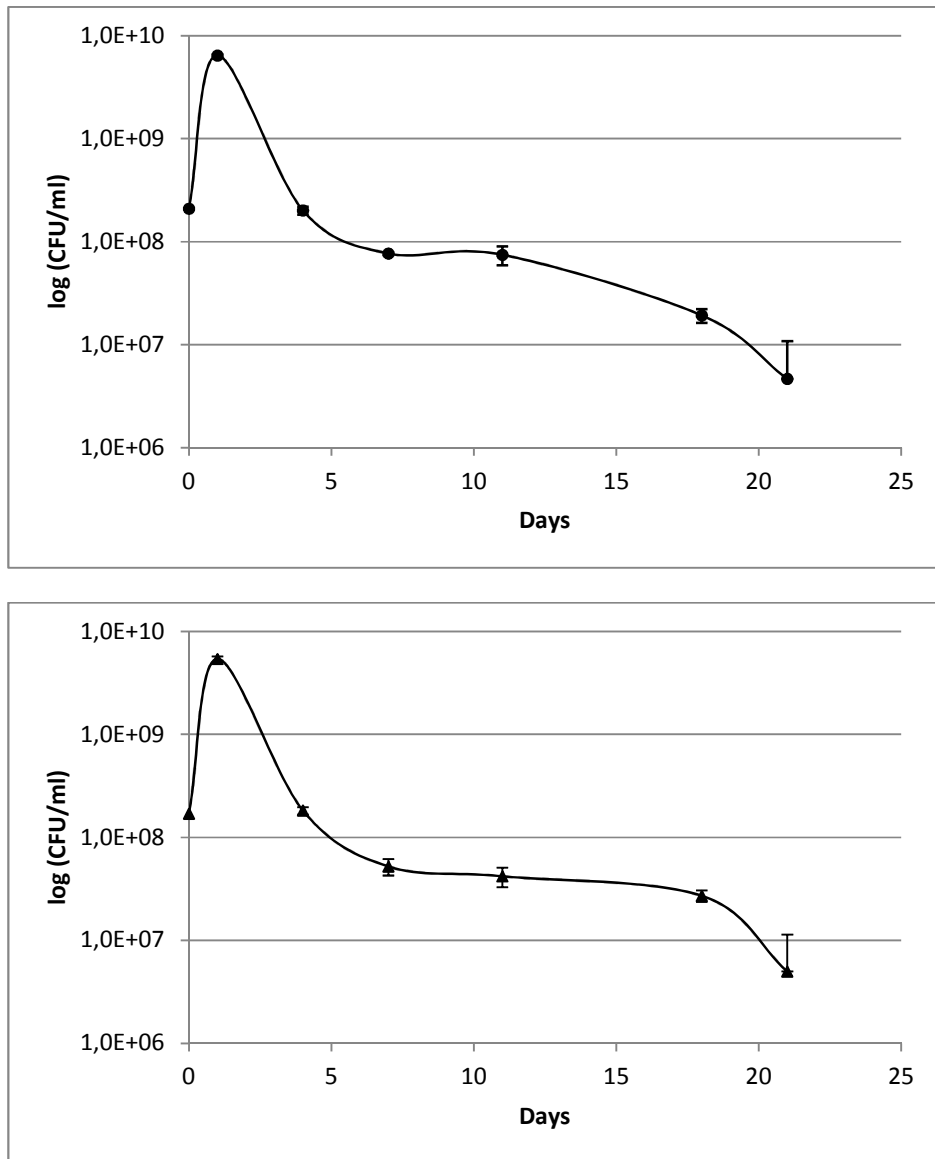
In order to compare the growth fitness of *C. jejuni*  $\Delta dps$  mutant with that of the wild type strain *C. jejuni* 11168, a growth experiment was performed in which both strains were grown separately in BHI medium for 80 hours (Figure 5-1).



**Figure 5-1. Growth of *C. jejuni* 11168 (circles) and *C. jejuni* 11168  $\Delta dps$  (triangles) at microaerobic conditions.** Both strains were inoculated separately into BHI medium to  $OD_{600}=0.05$ . Cultures were incubated in microaerobic conditions at 37°C for 80 hours. Samples were taken out at different time intervals and CFU/ml determined.

Both strains grew at a similar rate under the conditions tested (Figure 5-1). The most rapid exponential growth took place within the first 5 hours of growth, whereas the stationary phase peaked after approximately 24 hours. These two time points were considered as representative of each growth phase and used in the experiments described in Manuscript I.

In *Listeria monocytogenes* presence of the Dps homologue Fri in the cells gives them an advantage during long term growth compared to the wild type (Olsen et al. 2005). We investigated whether *C. jejuni* Dps could also improve growth of the cells in late stationary phase by comparing growth of *C. jejuni* 11168 with that of *C. jejuni*  $\Delta dps$  for the period of 21 days.

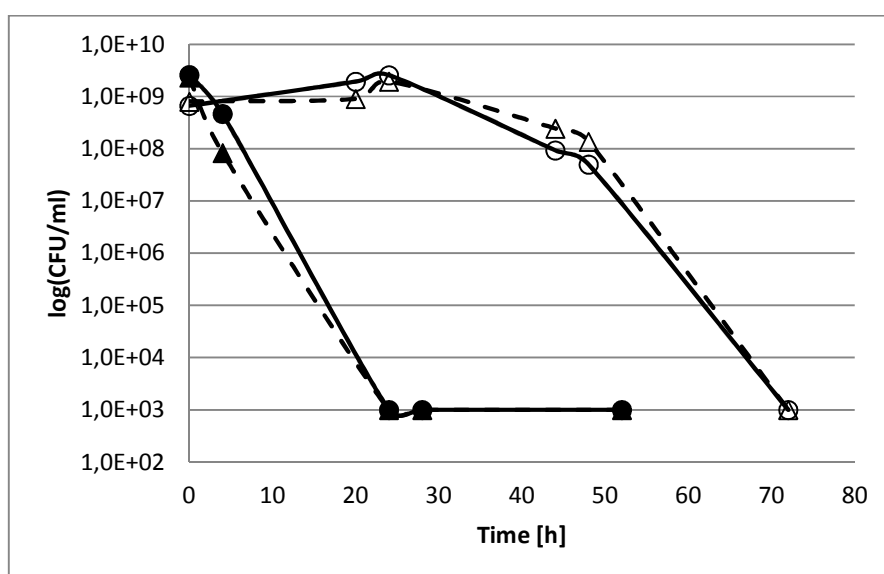


**Figure 5-2. Long term growth of *C. jejuni* 11168 (circles) and *C. jejuni* 11168  $\Delta$ dps (triangles) in microaerobic conditions.** Both strains were inoculated separately into BHI medium to OD<sub>600</sub>=0.05. Cultures were incubated in microaerobic conditions at 37°C for 21 days. Samples were taken out at different time intervals and CFU/ml determined. Standard deviation was calculated from 3 biological replicates.

No difference was observed in the long term growth of both strains which suggested that in *C. jejuni* Dps does not give an advantage to growing cells in any of the growth phases investigated (Figure 5-2).

## 5.2 Survival after prolonged incubation in aerobic conditions

In an attempt to assess whether *C. jejuni* Dps is required for survival under oxidative stress caused by aerobic conditions, the survival of *C. jejuni* 11168 and *C. jejuni*  $\Delta$ dps mutant under these conditions was compared in both exponential and stationary phases. Shortly, both strains were grown to exponential (4 h) and stationary (24 h) growth phases and then placed in aerobic conditions at 37°C and samples were taken several times until cell counts reached the detection limit ( $<10^3$  CFU/ml) (Figure 5-3).



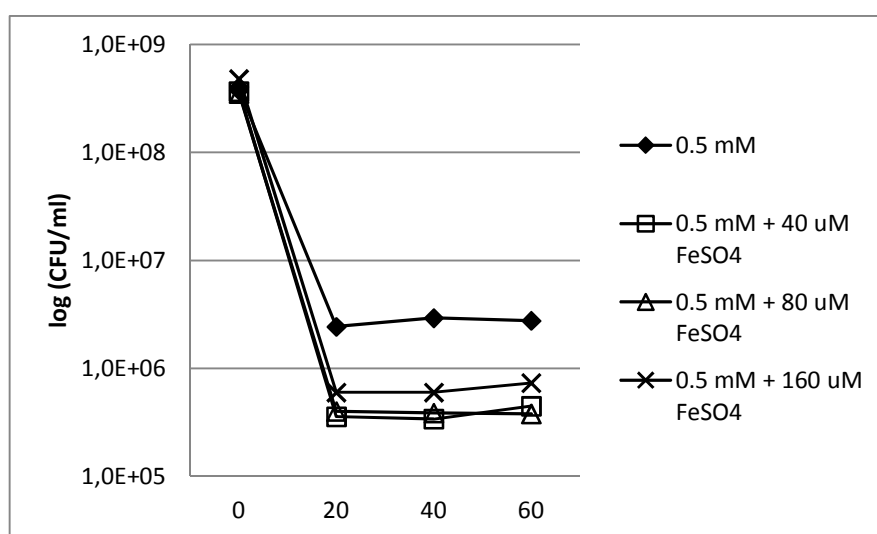
**Figure 5-3. Long term survival of *C. jejuni* 11168 and *C. jejuni* 11168  $\Delta$ dps in exponential and stationary growth phases in aerobic conditions.** *C. jejuni* 11168 (solid line, circles), *C. jejuni* 11168  $\Delta$ dps (dashed line, triangles), exponential phase (empty symbols), stationary phase (filled symbols). Both strains were grown in BHI medium to exponential (4 h) and stationary (24 h) growth phases and then placed in aerobic conditions at 37°C. Cultures were incubated and samples collected at different time intervals for CFU/ml determination until cell counts reached the detection limit ( $<10^3$  CFU/ml).

The presence of Dps protein in the wild type strain did not give any survival advantage compared to the strain lacking Dps (Figure 5-3). Both strains were more sensitive to aerobic incubation in stationary phase than in exponential phase. Such behavior was observed before for *C. jejuni* 11351, proving that this strain lacks a typical stationary phase response that would make the cells more resistant to stress (Kelly et al. 2001).



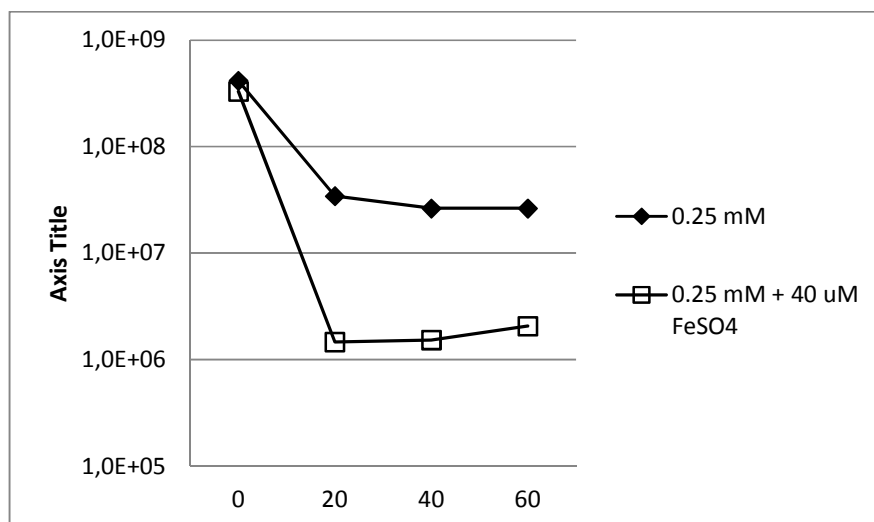
### 5.3 Effect of iron on growth and stress resistance

In Manuscript I, iron-limited medium MEM- $\alpha$  was used for investigating transcript and protein levels from the *dps* gene. Before conducting the experiments, I checked whether wild type and  $\Delta dps$  mutant cells grown in this medium display the same sensitivity to hydrogen peroxide as when they are grown in iron-rich medium. When that was confirmed (data not shown), I investigated the effect of combined treatment with different concentrations of hydrogen peroxide and iron source ( $\text{FeSO}_4$ ) only in the  $\Delta dps$  mutant from exponential phase, as this was the only strain where decrease in cell numbers was observed.



**Figure 5-4. Survival of *C. jejuni*  $\Delta dps$  mutant from exponential phase after exposure to 0.5 mM  $\text{H}_2\text{O}_2$  and varying  $\text{FeSO}_4$  concentrations.**

At 0.5 mM  $\text{H}_2\text{O}_2$  concentration a 1-log difference was observed with the bacteria being more sensitive to oxidative stress when additional iron is added (Figure 5-4). This indicates, as expected, that more hydroxyl radicals are formed as a result of Fenton reaction and therefore the damage is greater. However, the damage does not increase with the increasing amount of iron added. For this reason I decided to use 40  $\mu\text{M}$   $\text{FeSO}_4$  in the experiments described in Manuscript I.

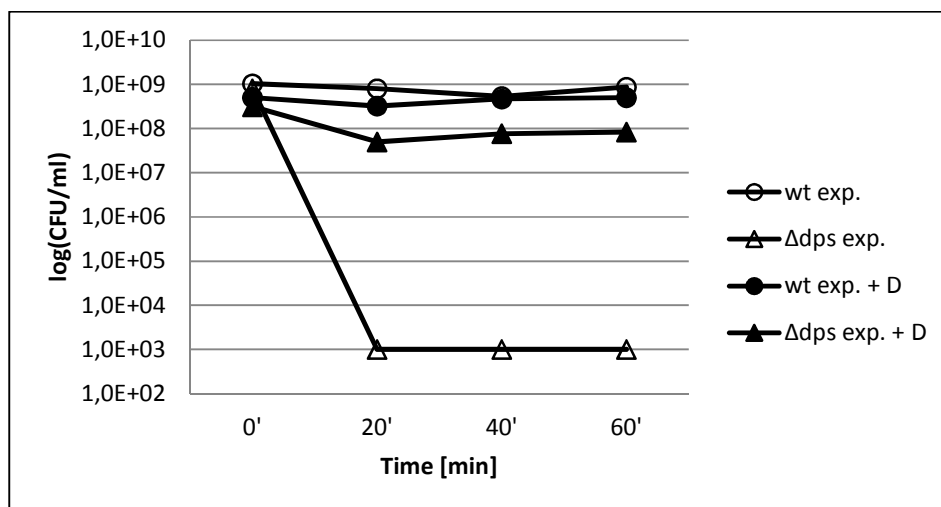


**Figure 5-5. Survival of *C. jejuni*  $\Delta dps$  mutant from exponential phase after exposure to 0.25 mM H<sub>2</sub>O<sub>2</sub> and varying FeSO<sub>4</sub> concentrations.**

A similar difference in survival was observed at 0.25 mM H<sub>2</sub>O<sub>2</sub>, depending on whether or not the iron source was added (Figure 5-5).

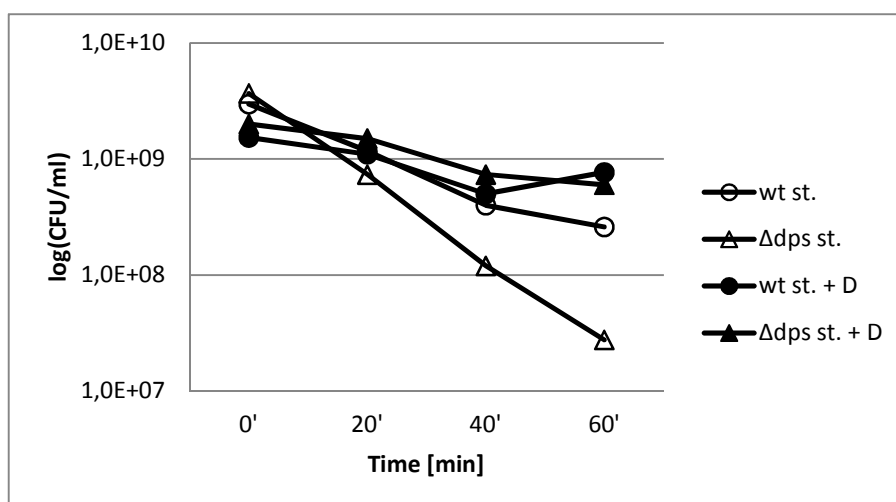
#### **5.4 Does iron binding prevent stress in exponential phase?**

The iron-binding property of *C. jejuni* Dps was shown previously (Ishikawa et al. 2003). Additionally, it was shown that the resistance of the  $\Delta dps$  strain to oxidative stress can be restored by pretreating the strain with an iron chelator Desferal which indicated that the ability of Dps to bind iron could be the main protection mechanism against oxidative stress. In our study we compared the effect of the same iron chelator on the sensitivity to oxidative stress during exponential and stationary phases in order to investigate whether Dps might use another mechanism in exponential phase than in stationary phase to protect the cells from decrease in survival. Shortly, the wild type and the  $\Delta dps$  mutant strains were grown to exponential and stationary growth phases and half of the samples were pretreated with Desferal.



**Figure 5-6. Effect of pretreatment of *C. jejuni* 11168 and *C. jejuni*  $\Delta$ dps mutant in exponential phase with an iron chelator.** The cultures were pretreated with 80mM Desferal for 5 min before exposure to 2.5mM  $H_2O_2$  incubated for 1 hour at 37°C in microaerobic conditions.

When the exponential phase culture is pretreated with Desferal prior to stress, we can observe almost complete restoration of the wild type phenotype in the *dps* mutant (Figure 5-6). This indicates that when iron is bound, no hydroxyl radicals can be formed through the Fenton reaction and therefore no damage to the cells can occur even in the absence of Dps.



**Figure 5-7. Effect of pretreatment of *C. jejuni* wt and *dps* mutant in stationary phase with 80mM Desferal on sensitivity to 2.5mM  $H_2O_2$ .**

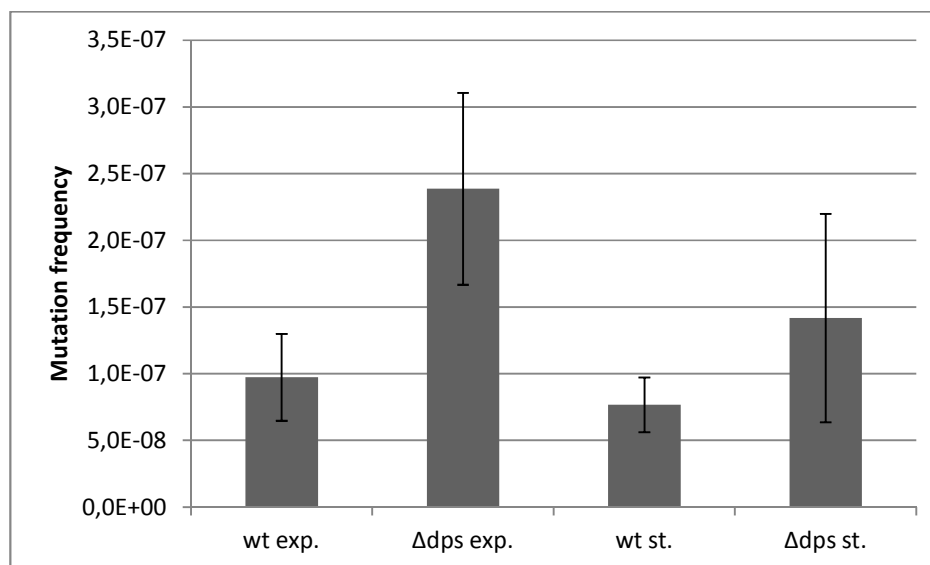
In stationary phase, even though the decrease in cell numbers caused by hydrogen peroxide is not so pronounced, we can still observe a complete restoration of the wild type phenotype when

iron chelator is added (Figure 5-7). This indicates that, indeed, the iron binding ability of Dps alone is enough to prevent oxidative damage also to the exponential phase cells.

## 5.5 Mutation frequency

Mutation frequency was determined in the wild type *C. jejuni* strain and the  $\Delta dps$  mutant in order to check if the values differ depending on the growth phase and the presence of Dps. Increased mutation frequency values in the  $\Delta dps$  mutant in any of the growth phases compared to the wild type could indicate increased DNA mutation caused by the lack of Dps. In order to obtain spontaneous mutants, plates with 100 µg/ml nalidixic acid were used. Nalidixic acid is a quinolone, which acts by inhibiting DNA synthesis by targeting two essential type II topoisomerases, DNA gyrase and topoisomerase IV (Fàbrega et al. 2009).

The experiment was performed as described before (Björkholm et al. 2001), with modifications. Ten independent *C. jejuni* wild type cultures and 10 independent  $\Delta dps$  mutant cultures were grown to exponential (4 h) and stationary (24 h) phases in microaerobic conditions at 37°C. A viable count determination was performed on three of the cultures representative of each strain and growth phase. One ml of each stationary phase cultures and 4 ml of each exponential phase cultures was concentrated by centrifugation and resuspended in 100 µl BHI and the whole volume was spread on Base II plates containing Nalidixic acid (100 µg/ml). The plates were then incubated for 3 to 6 days in microaerobic conditions and the number of mutants was recorded. Mutation frequency was calculated as the mean number of mutants from 10 cultures divided by the mean of the three viable counts (Figure 5-8).



**Figure 5-8. Mutation frequency of *C. jejuni* wild type and  $\Delta dps$  mutant strain from exponential and stationary growth phases.** Error bars represent standard deviation. n=10.

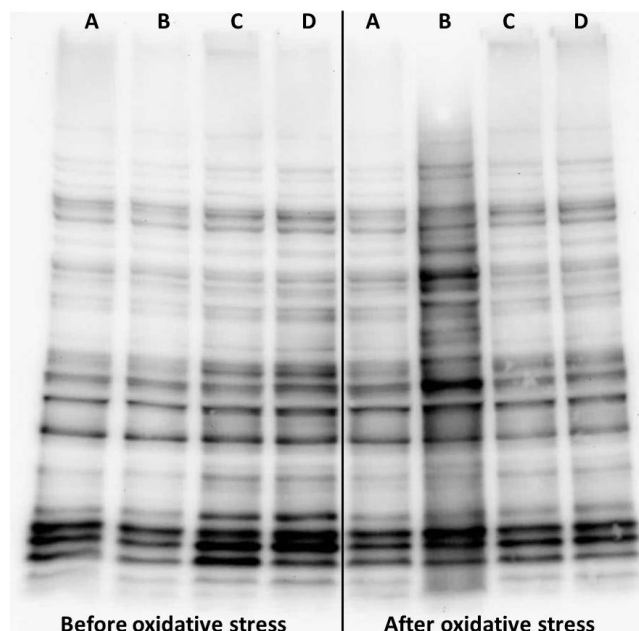
The results presented in Figure 5-8 show that the  $\Delta dps$  mutant had slightly increased mutation frequency, especially when the cells were in exponential phase compared to the wild type (2.5-fold increase in mutation frequency in the  $\Delta dps$ ), but also in stationary phase (1.8-fold increase in the  $\Delta dps$ ). Even though the experiment should be repeated with a higher number of cultures (n=20), there is a certain indication that *C. jejuni* DNA is more prone to mutations in the absence of Dps in both exponential and stationary phase. The slightly increased mutation frequency of the strains in the absence of Dps could also indirectly indicate that in the wild type cells Dps-DNA interaction could occur.

## 5.6 Detection of carbonylated proteins

Protein damage resulting from oxidative stress occurs in a process known as carbonylation, which is an irreversible protein modification caused by accumulation of oxygen radicals (Nystrom 2005). Carbonylation can be detected in the cells and used as an estimation of the level of oxidative damage.

Wild type and  $\Delta dps$  mutant strains in exponential and stationary phases were exposed to 2.5 mM  $H_2O_2$  for 1 hour. Culture samples were taken before exposure and 60 minutes later, protein extracts collected and carbonyl groups derived. Samples were run on a SDS-PAGE gel and carbonyl groups were detected using anti-DNP antibodies from the kit. After 60 minutes of oxidative stress,

heavy carbonylation was observed in the mutant in exponential phase after stress exposure compared to other samples (Figure 5-9).



**Figure 5-9. Protein carbonylation in *C. jejuni* 11168 and *C. jejuni*  $\Delta dps$  in different growth phases before and after exposure to 2.5mM  $H_2O_2$  for 1 hour.** Wild type exponential (exp.) phase (A),  $\Delta dps$  exp. phase (B), wild type stationary (st.) phase (C),  $\Delta dps$  st. phase (D).

This result indicates a high level of protein damage in the absence of Dps in exponential phase cells, underlining the protein's role during stress in this growth phase. It has to be noted that after 60 minutes exposure to 2.5 mM  $H_2O_2$  the cell counts in the  $\Delta dps$  mutant from the exponential phase cells were very low, beyond the detection limit ( $>1000$  CFU/ml). Therefore the large level of carbonylation seen in Figure 5-9 could be due to the fact that a lot of cells are dead.

## 5.7 Detailed method description – sample collection and derivatization of carbonyl groups

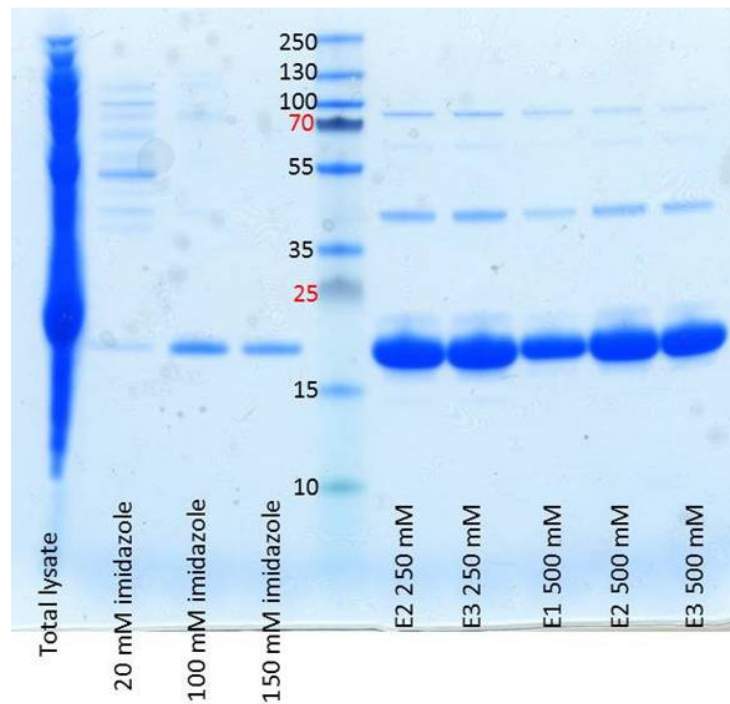
Fresh cultures of *C. jejuni* 11168 and *C. jejuni* 11168  $\Delta dps::cat$  were grown overnight on Base II plates. They were then inoculated into 20 ml BHI to an  $OD_{600}=0.05$  and grown to exponential (4h) and stationary (24h) phases. When the desired growth phases were reached, 10 ml of each culture was removed,  $OD_{600}$  was measured and the culture was harvested at  $6000 \times g$  for 10 minutes. Hydrogen peroxide was added to the remaining volume of cultures to a desired concentration (2.5 mM) and the strains were incubated in microaerobic conditions at  $37^\circ C$  for 1

hour. The previously harvested pellet was resuspended in freshly prepared lysis buffer (PBS, 8 M urea, 1%  $\beta$ -mercaptoethanol, 1% Triton X-100, 1% SDS) to obtain  $OD_{600} = 5$  and incubated with shaking for 1 hour at room temperature. The lysate was then sonicated, followed by a centrifugation at  $10000 \times g$  for 10 minutes. The supernatant containing total protein was stored at  $-20^{\circ}\text{C}$  for further use. The samples subjected to oxidative stress were treated in the same way after 1 hour of incubation.

The protein concentrations were measured by densitometry of proteins stained with Amido Black on a nitrocellulose membrane, using Bovine Serum Albumin (BSA) as a standard. The carbonyl groups were derived using the OxyBlot™ Protein Oxidation Detection Kit essentially according to manufacturer's instructions but with a few modifications. Double amount of sample and of every solution was used and  $\beta$ -mercaptoethanol was not added. The proteins were then separated on a 10% BT 10-well SDS gel in MES buffer (Invitrogen) and transferred to a PVDF membrane using electroblotting. Western blot detection was performed essentially as described in the manual, using the Immobilon Western HRP substrate (Milipore). Primary antibody was diluted 1:150 and the secondary antibody 1:300. Samples were visualized using Geliance 1000 Imaging System (Perkin Elmer).

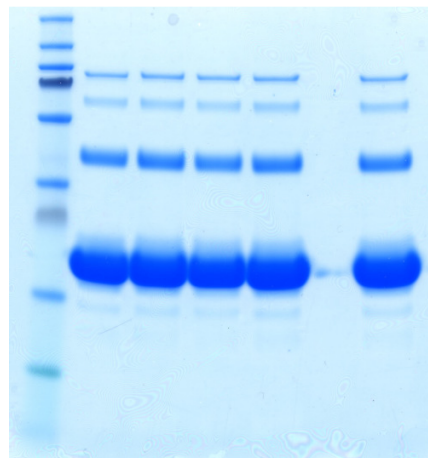
## **5.8 Dps purification**

For the description of Dps protein purification, see Materials and Methods in Manuscript I. Here, gel pictures are presented showing the size of the protein and the additional bands present in each sample.



**Figure 5-10. Dps protein elution fractions.** *C. jejuni* Dps protein size is 17 kDa.

During Dps protein purification, increasing concentrations of imidazole were used in the elution buffer in the hope of getting rid of the additional bands, larger than the Dps protein, present in each elution fraction (Figure 5-10). The larger bands do not match sizes that could indicate dimers or higher oligomers of the Dps protein. It was not possible to remove the additional bands, therefore, the gel fragment containing the Dps protein was excised from the gel and used for rabbit immunization (Figure 5-11).



**Figure 5-11. Dps protein sent for immunization.**



## **Chapter 6**

### **Discussion and perspectives**

The research performed during this PhD project has led to a better understanding of ways in which *Campylobacter jejuni* adapts to a variety of environments that it encounters. In this chapter the most important findings are summarized and further discussed, taking into account supplementary data. Concluding remarks are then followed by future experiments suggestions.

## 6.1 Investigating growth phase differences

The study presented in Manuscript I focused on the observed differences in stress resistance of *C. jejuni* between exponential and stationary growth phases. Based on an example of the oxidative stress protein Dps that in other Gram negative bacteria is known to be induced upon entry into stationary phase (Almiron et al. 1992;Altuvia et al. 1994), we showed how regulation and sensitivity to oxidative stress differs between these two growth phases in *C. jejuni*. In contrast to the findings from other Gram negative bacteria where the Dps homologues were induced in stationary phase and, as a result, cells in this growth phase were more resistant to different types of stress (Almiron et al. 1992;Cooksley et al. 2003;Yoo et al. 2007), our data showed that in *C. jejuni* the cells in exponential phase lacking Dps protein were more sensitive to oxidative stress than stationary phase cells (Figure 2-1).

Our data have not given a clear answer as to why the exponential phase cells lacking Dps protein were more sensitive to peroxide stress. We have shown that the transcripts of several other oxidative stress genes such as *kataA*, *tpx*, *ahpC* and *sodB* are either present or can be induced in exponential growth phase and that could be helpful to the cells in preventing the toxic effect of hydrogen peroxide (Figure 2-8), however, it seems not to be enough.

Even though *C. jejuni* lacks *rpoS* gene and thereby does not show a typical stationary growth phase response (Schellhorn et al. 1998), selected physiological changes do take place in *C. jejuni* cells upon entry into stationary phase, such as increase in cellular fatty acid composition, greater physical resistance of the membrane to pressure as well as increase in cellular pressure resistance (Martinez-Rodriguez and Mackey 2005). The stationary phase of *C. jejuni* was also described as dynamic in another study, where certain changes such as high motility as well as changes in gene expression and switching in substrate utilization and production were observed (Wright et al. 2009). However, no genetic mechanism has been pointed out as responsible for the observed changes related to stationary phase.

It is possible that the above mentioned physiological changes could lead to increased resistance to peroxide stress also in our  $\Delta$ dps mutant in stationary phase. Assuming that this indeed is the case, these changes are only effective for resistance against peroxide stress in the  $\Delta$ dps

mutant, and not aerobic conditions, since we did not observe differences in sensitivity to aeration between the mutant and the wild type (Supplementary results, Figure 5-3). What is more, the exponential phase cultures in general were more resistant to aeration than the stationary phase cells (Supplementary results, Figure 5-3), which is in agreement with others (Kelly et al. 2001). In contrast, the same cells exposed to cumene hydroperoxide had greater sensitivity in exponential phase (wild type and  $\Delta dps$  mutant alike) than in the stationary phase. It seems therefore that in addition to growth phase-based differences in response to stress, the type of ROS to which *C. jejuni* is exposed also plays an important role.

To sum up, the sensitivity of the  $\Delta dps$  mutant to peroxide stress is most likely due to accumulation of hydroxyl radicals to a level that is too toxic for the cells in exponential phase lacking the Dps protein. The fact that the  $\Delta dps$  mutant in stationary phase is more resistant to the same level of peroxide stress could be a result of physiological changes such as increase in cellular fatty acid composition and physical membrane resistance, that contribute to making the cell more resistant in this growth phase or an unknown peroxide response mechanism specific to this growth phase.

Even though the exponential phase cells lacking Dps were more sensitive to peroxide stress than the stationary phase cells, we observed an increased level of the Dps protein in a 24 hour old culture compared to a 4 hour old culture (Figure 2-5). We suspect that the increased amount of Dps protein in stationary phase sample was not due to the stationary phase induction of the protein, which is not likely because of the lack of RpoS (Lange and Hengge-Aronis 1991) in *C. jejuni* (Parkhill et al. 2000), but rather simply accumulation of the protein in the cells towards stationary growth phase. Alternatively, induction could be possible by means of a different growth phase dependent regulator.

In fact, there is one known type of stationary phase response that has been identified in *C. jejuni* and that is the stringent response typical for other Gram negative bacteria (Jain et al. 2006). Induced by environmental signals such as starvation, it leads to global changes in gene regulation that help the bacterium survive challenging conditions (Chatterji and Ojha 2001; Magnusson et al. 2005). It is thought that lack of nutrients leads to accumulation of uncharged tRNA molecules at the ribosomal acceptor site, which causes stalling of the ribosome and activation of a ribosomal bound protein RelA or SpoT. As a result, synthesis of guanosine pentaphosphate (pppGpp) is catalyzed, which is subsequently hydrolyzed to guanosine tetraphosphate (ppGpp) that binds RNA polymerase and can affect gene expression at the transcriptional level, but also influence DNA replication, recombination and repair (Chatterji and Ojha 2001). In Gram negative bacteria both

RelA, synthesizing pppGpp, as well as SpoT, degrading (p)ppGpp are needed for the functional stringent response, whereas in Gram positive bacteria, a single *relA/spoT* homologue is required for the same purpose (Gaynor et al. 2005; Mittenhuber 2001). A number of functions have been reported to be regulated by the stringent response in different pathogens and they are nicely reviewed by others (Dalebroux et al. 2010). Apart from increased stationary phase survival, where the  $\sigma^S$  factor is in fact positively regulated by ppGpp (Gentry et al. 1993), these functions also include adherence, colonization, quorum sensing, biofilm formation, antibiotic resistance and many more.

The genome of *C. jejuni* contains a *spoT* gene, highly homologous to *relA* and *spoT* genes in other bacteria (Gaynor et al. 2005). The gene in *C. jejuni* was reported to be rapidly upregulated during infection of human epithelial cells and an investigation of a  $\Delta spoT$  mutant revealed that the gene is required for survival during stationary phase, survival and growth under low CO<sub>2</sub>/high O<sub>2</sub> conditions, as well as rifampicin resistance. A number of genes was identified that had differential gene expression in the  $\Delta spoT$  mutant compared to the wild type, however, none of them were the genes investigated in this thesis. The stringent response in *C. jejuni* was therefore most likely not the reason for our observed phenotype of increased sensitivity to peroxide stress in the  $\Delta dps$  mutant. Nevertheless, the existence of the stringent response in *C. jejuni* as the only identified and described stationary phase-dependent gene induction system indicates that the growth phase regulation in this pathogen is different from that of other bacteria and still has many unknowns.

In general, when investigating differences between growth phases on any level, one has to keep in mind the differences between laboratory conditions and the conditions that the bacterium encounters in the environment. What bacteria experience in the environment should be viewed as occasional periods of exponential growth, where nutrients are readily available, mixed with periods of stationary phase in which the bacteria must deal with nutrient deprivation (Kolter et al. 1993; Rees et al. 1995). The starvation periods are most likely encountered by bacteria more often than the periods with abundant nutrients, nevertheless, the exponential growth periods must have occurred in the past often enough for the bacteria to evolve regulation mechanisms specific for this growth phase. One example of a regulation mechanism occurring only in exponential phase is the previously mentioned control of *dps* in *E. coli*, where the protein is induced upon stress by OxyR in the exponential phase, whereas in stationary phase it is  $\sigma^S$  that induces the protein, in spite of presence of OxyR in that growth phase (Altuvia et al. 1994).

## **6.2 Role of regulators PerR and Fur is growth phase dependent**

The data presented in Manuscript I indicate that the PerR and Fur regulators have different effect on Dps regulation depending on the growth phase of *C. jejuni*. Whereas in exponential phase both regulators act as repressors, in stationary phase Fur still acts as a repressor, however, PerR likely becomes an activator in the absence of Fur (Figure 2-6, Figure 2-7). Growth phase-dependent activation of a regulator was already described in *C. jejuni* (Wösten et al. 2010), where a two-component system DccRS was induced in late stationary growth phase, positively influencing colonization of chickens. Again, no genetic counterparts were identified that could cause this induction, instead, an experiment with exchanging media suggested that an unknown secreted factor accumulating in the stationary growth phase could be the trigger for activation of DccRS regulon, but not the DccR (cognate response regulator) itself. Even though the authors of that study do not suggest it, one could speculate that the unknown factor could be produced as a result of ppGpp induction. It was reported for other pathogens that ppGpp influences, among many other factors, production of exoproteins, for example the quorum sensing molecules in *Pseudomonas aeruginosa*, that are necessary for this pathogen's virulence (Erickson et al. 2004).

One could speculate that a kind of stimulus present only in stationary phase could lead to change in roles of the two repressors. The proteins PerR and Fur certainly play an important role in regulation of the Dps protein and the fact that their role differs between growth phases only shows the complexity of this regulation.

## **6.3 *Campylobacter jejuni* thrives in chicken meat environment**

In Paper I we showed that *C. jejuni* has a limited but specific transcriptional response to chicken meat juice, an environment that greatly facilitates this pathogen's survival (Birk et al. 2004).

Among the genes found to change their expression in the chicken meat juice environment compared to BHI medium, none of them was encoding a known regulatory protein, indicating that induction of these genes was rather caused by environmental signals from the chicken meat juice than a specific regulator. The upregulation of a part of the putative iron uptake system (*cfbpc*) confirms that speculation.

## 6.4 Genetic tools for *Campylobacter jejuni* are still in need

Since the publication of the *C. jejuni* 11168 genome in 2000 (Parkhill et al. 2000), many genetic tools were developed that enabled more advanced studies on this enigmatic pathogen. These included random and signature-tagged transposon mutagenesis (Colegio et al. 2001; Golden et al. 2000; Grant et al. 2005), a genetic system designed for construction of defined deletions in the chromosome (Hendrixson et al. 2001), that was also used in our study, as well as DNA microarrays (Carrillo et al. 2004; Dorrell et al. 2001).

Concluding the numerous attempts of constructing *C. jejuni* mutants in the *clp* genes by well-established methods undertaken during the work summarized in Supplementary Results I, I believe that more genetic tools, such as various inducible expression vectors in *C. jejuni* are still required, and it would certainly be useful if scientists could report their negative experiences with the existing methods. Furthermore, very careful considerations must be taken before constructing mutants in *C. jejuni* that could possibly save time, such as the previously discussed possibility of minimizing recombination events in the *cat-rpsL* method (Hendrixson et al. 2001) by using genes from *H. pylori* that have high protein sequence homology, but lower DNA sequence homology (Dailidienė et al. 2006).

## 6.5 Perspectives

### The growth phase-dependent regulation and the Dps protein explored further

The work presented in this study could be continued in a number of ways. An obvious way to continue the story described in Manuscript I would be to investigate in more depth the post-transcriptional or post-translational regulation of the Dps protein in different growth phases. I imagine that such a project would focus on the strains lacking the PerR and Fur regulators and could perhaps include the two other regulators that recently have been described to be involved in regulation of the Dps protein in connection with oxidative stress, namely the CosR regulator (Hwang et al. 2011) and Cj1556 (Gundogdu et al. 2011). The post transcriptional regulator CsrA, already found to have a role in oxidative stress in *C. jejuni* (Fields and Thompson 2008), would also be a desired research target in this field.

A good starting point for such analysis would be to determine precisely the *dps* transcript and Dps protein level in all the above mentioned mutants in different growth phases, using quantitative methods, if possible, such as qPCR instead of, or in addition to, Northern blots for the transcript levels and Western blot, which is a semi quantitative method, for the protein levels.

### **Dps in *C. jejuni* – what else can we discover?**

Another line of research could be to focus on the Dps protein from *C. jejuni* and on what other possible roles it might have. In many other bacteria, the Dps protein is capable of unspecifically binding DNA (Almiron et al. 1992; Chen et al. 1995; Gupta and Chatterji 2003), thereby protecting it from oxidative damage. The DNA-binding ability of Dps was so far only investigated in one study, which concluded that *C. jejuni* Dps is not capable of binding plasmid DNA *in vitro* (Ishikawa et al. 2003). While the study is convincing, in my opinion additional experiments could be performed to ensure that DNA binding by Dps really does not occur in *C. jejuni*. It is possible that in this pathogen, DNA binding might only occur *in vivo*, in a specific growth phase or under specific stress conditions and to investigate that I would suggest looking into the cells isolated under different conditions by means of, for example, Atomic Force Microscopy (AFM). If Dps can indeed bind DNA, it would be condensed. Alternatively, more *in vitro* experiments could be performed, but this time using the mixture of purified, native Dps protein and DNA in the presence of other factors such as H<sub>2</sub>O<sub>2</sub>, iron or other divalent cations.

The fact that Dps is able to bind DNA in other bacteria also leads to the question of whether in this way Dps could affect regulation of other genes. This would, however, require binding of the protein to specific sequences on the genome and this ability has not been demonstrated for Dps so far. On the other hand, it is easy to imagine that when DNA is tightly bound by Dps (Frenkiel-Krispin et al. 2004), it is unlikely that RNA synthesizing enzymes or others could bind to it as well. In *E. coli* large differences were reported between the proteome of the wild type and the  $\Delta$ dps mutant in late stationary phase, indicating that Dps could affect the proteome (Almiron et al. 1992). It would be therefore interesting to examine whether there are differences between the global gene expression as well as the proteome of the *C. jejuni* wild type and the  $\Delta$ dps mutant.

In order to check whether Dps has a role in chicken colonization, the study could be supplemented with colonization experiments involving the wild type and the  $\Delta$ dps mutant. In fact, very recently it was demonstrated that *C. jejuni* Dps was required for colonization in new born piglets, as well as it was significantly upregulated in the wild type strain during colonization and survival in the host (Theoret et al. 2011). It would certainly be useful to perform colonization experiments in chickens, which are the main reservoir of *C. jejuni* that can affect humans.

### **Mysteries of the chicken meat juice**

When investigating the gene expression in chicken juice, I was missing information about the precise composition of the chicken meat juice. Certainly this composition would differ from one

chicken meat piece to another, nevertheless, some common compounds such as lipids or proteins could surely be identified. Assuming it would be possible to identify at least partially these compounds, survival experiments could then be performed at low temperatures, where wild type *C. jejuni* could be incubated with one compound at a time or a combination of chosen compounds. Hopefully this approach could lead to identification of specific compounds which are responsible for *C. jejuni* survival. Knowledge of such compounds could lead to development of strategies that would be directed specifically at deactivating this compound on chicken meat and thereby decreasing the chances of *C. jejuni* survival on meat products.

The data presented in Manuscript II could be nicely supplemented with a study investigating the survival of the mutants in the genes upregulated in the chicken meat juice compared to BHI. This would help determine whether other genes, apart from *luxS*, are also equally important for survival in this important environment.

## 6.6 Concluding remarks

All in all, the work presented in this thesis has shed more light on how *Campylobacter jejuni* adapts to oxidative stress and poultry meat environment, both conditions occupying quite significant roles in this pathogen's life. Even though much is known about how *C. jejuni* copes with oxidative stress, more research is still needed to establish the details of stress sensing and response. This work has underlined that the response to stress in this pathogen differs depending on the growth stage and should therefore be looked at separately in each growth phase. Dps is an excellent example of a growth-phase regulated stress protein and increasing evidence is accumulating that underlines its importance in various aspects of *C. jejuni* life, including virulence. At the same time in our work we got one step closer to determining what is helpful for this pathogen to survive for long periods in the chicken meat environment. *Campylobacter jejuni* is certainly an enigmatic and unique pathogen and further research combined with the use of new genetic tools will surely reveal more surprises and new discoveries.



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